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Analysis of anthocyanins in strawberries and elderberries. A comparison of capillary zone electrophoresis and HPLC

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Anthocyanins in strawberry and elderberry extracts were separated by reverse phase HPLC at pH 1·8, and compared with separations achieved by capillary zone electrophoresis using a standard silica capillary and pH 8·0 running buffer. HPLC separated all the anthocyanins in both extracts, although the similar characteristics of the anthocyanins in the elderberry extract made this the more difficult separation. CZE separation of strawberry anthocyanins was of a quality comparable with results obtained with a standard anthocyanin mixture. Elderberry pigments gave a poorer electropherogram, possibly due to additional interfering compounds in the extract. A greater sample concentration (87 times) was required for equivalent CZE response compared with HPLC, due mainly to the much smaller introduction volume, the shorter detector cell path-length and the small proportion of coloured anthocyanin species present at pH 8·0. The results indicate that under these conditions, HPLC has more advantages, but CZE has potential, particularly if methodology for working with strongly acidic buffers becomes available. Published by Elsevier Science Ltd

INTRODUCTION

HPLC is well established for most aspects of food analysis (Macrae, 1988) and it is the standard method for qualitative and quantitative analysis of anthocyanins (e.g. Strack & Wray, 1989); it is also suitable for semi-preparative work. Strawberry (*Fragaria ananassa*) pigments were analysed by HPLC (Hong & Wrolstad, 1990*a*) with positive identification of two of the five anthocyanin peaks which were observed. HPLC analysis in our laboratory of 39 named strawberry genotypes (Bakker *et al.*, 1994), showed a range of up to 13 anthocyanin peaks, depending on genotype. Of these, pelargonidin 3-glucoside, pelargonidin 3-rutinoside and cyanidin 3-glucoside were positively identified, and cyanidin and pelargonidin 3-succinylglucosides were tentatively identified from subsequent FAB-MS data. Recently, a new strawberry pigment (pelargonidin 3-malonylglucoside) was identified in the Japanese cultivar 'Nyoho' (Tamura *et al.*, 1995).

Elderberry (*Sambucus nigra*) anthocyanins are all based on cyanidin, (3-glucoside, 3-sambubioside, 3-sambubioside-5-glucoside and 3,5-diglucoside) (Bronnum-Hansen & Hansen, 1983). Additionally, acylated pigments have been reported in other elderberry species (Lamaison *et al.*, 1979; Johansen *et al.*, 1991; Nakatani *et al.*, 1995). It appears that the qualitative and quantitative distribution of pigments is dependent on the variety studied (Mazza & Miniati, 1993). The satisfactory separation by HPLC of the four basic pigments has posed some problems; certain solvent systems used previously provided poor resolution of either the 3-glycosides (Johansen *et al.*, 1991) or the 3,5-diglycosides (Hong & Wrolstad, 1990*b*); the introduction of tetrahydrofuran into the solvent system improved the separation (Bronnum-Hansen & Hansen, 1983; Drdak & Daucik, 1990).

Capillary electrophoresis is still a comparatively new technique; the capabilities of this technique for the analysis of a wide variety of compounds have been described (Kuhr & Monnig, 1992) and its suitability for the separation of phytochemicals, including the flavonoids, has been demonstrated (Morin & Dreux, 1993). We have applied capillary zone electrophoresis (CZE) successfully for the analysis of non-coloured phenolics in red wines (Gil *et al.*, 1995) and compared it with HPLC for analysis of these compounds (García-Viguera & Bridle, 1995); others have compared these techniques for pharmaceuticals analysis (Altria & Rogan, 1994). More recently, probably the first analysis of a mixture of standard anthocyanins by CZE was reported (Bridle

et al., 1996). This work produced encouraging results, and it was of further interest to compare the capabilities of CZE and HPLC for the analysis of these important plant pigments in some fruit extracts.

MATERIALS AND METHODS

Preparation of extracts

Strawberry anthocyanins
'Senga sengana' strawberry juice, deep frozen from a previous study (Bakker *et al.*, 1994), was thawed and the anthocyanin pigments isolated and concentrated on a conditioned (methanol then aqueous 3% formic acid wash) Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA, USA). The loaded cartridge was thoroughly washed with 3% aqueous formic acid and the pigments eluted with 3% formic acid in methanol. The resulting solution was rotary evaporated to small volume at 40°C and finally dried *in vacuo* to yield an anthocyanin extract in a powdered form.

Elderberry anthocyanins
Air dried elderberries (*Sambucus nigra*) were soaked for several days in methanol containing 1% HCl. The strongly coloured liquor was concentrated by rotary evaporation to remove methanol, diluted with a little 3% aqueous formic acid; the anthocyanins were isolated and washed on a Sep-Pak cartridge and dried as described above.

HPLC

Anthocyanin samples were analysed using a Hewlett-Packard 1090 M Series II liquid chromatograph, equipped with a diode array detector (6 mm path length), an auto-injector (25 μL) and a ODS-Hypersil reversed phase column (200×2·1 mm, 5 μm), at 40°C and 0·3 m L min^{-1} flow rate. In each case, the solid extract was dissolved in the starting solvent mixture and filtered (0·45 μm) ready for injection. Gradient elution (pH 1·8) was used as follows.

Strawberry
Elution solvents were (A) aqueous 0·6% $HClO_4$ and (B) methanol, starting with 15% B rising to 55% B over 40 min, with detection at 502 nm, the visible absorbance maximum wavelength of the main strawberry pigment (Fig. 3).

Elderberry
Elution solvents were (A) aqueous 0·6% $HClO_4$ and (C) tetrahydrofuran, starting with 2% C rising to 30% C over 28 min, with detection at 520 nm, the wavelength of maximum absorbance of elderberry anthocyanins.

The minimum extract concentration for HPLC detection was determined using a weighed amount of powdered strawberry extract (4·6 mg) dissolved in the initial solvent mixture (10 mL), with progressive dilution until the minor peaks were not detectable in the resulting chromatogram.

CZE

A Beckman P/ACE system 5510 was used, with diode array detection (detector path length 75 μm) at 560 nm, the visible maximum absorbance wavelength (Fig. 3). The fused silica capillary was 57 cm total length (50 cm to detector)×75 μm i.d., run at 25°C with 150 mM sodium borate buffer (pH 8·0). Samples were introduced by hydrodynamic injection for 2 s (9 nL volume, calculated using the Poiseuille equation—Harbaugh *et al.*, 1990) and run at 25 kV, producing a current of approximately 30 μA. The system was configured to run from anode to cathode. Electropherograms were processed using Beckman System Gold PC-based chromatography data system software, version 8.11. The capillary was conditioned by washing with methanol for 5 min followed by freshly prepared 1 M sodium hydroxide (5 min), 0·1 M sodium hydroxide (5 min), distilled water (3 min) and fresh electrophoretic buffer (3 min). For optimal migration time and peak shape reproducibility, the capillary was flushed between analyses with 0·1 M sodium hydroxide (3 min) and distilled water (2 min).

Anthocyanins were dissolved in a mixture of 25 mM phosphate buffer (pH 2·5) and methanol (3:1) and filtered (0·45 μm) ready for analysis. A solution of 4 mg/mL was the minimum concentration required for satisfactory detection of the minor peaks in the strawberry extract.

Peak identities were confirmed by the addition of available standards to both extracts in both techniques.

RESULTS AND DISCUSSION

For comparison with CZE, we tested a relatively simple HPLC separation (strawberry anthocyanins) and a more difficult separation (elderberry anthocyanins). In the strawberry sample, we previously identified four main anthocyanins, but reported five other minor or trace peaks in a concentrated extract (Bakker *et al.*, 1994). For the purposes of this comparative study, only the four main anthocyanins were considered.

HPLC

Strawberry anthocyanins were well resolved (Fig. 1a). The most polar compound, cyanidin 3-glucoside (1) eluted first, followed by the main anthocyanin pelargonidin 3-glucoside (2), pelargonidin 3-rutinoside (3) and the least polar pigment pelargonidin 3-succinylglucoside (4), in accordance with theory for reverse phase HPLC.

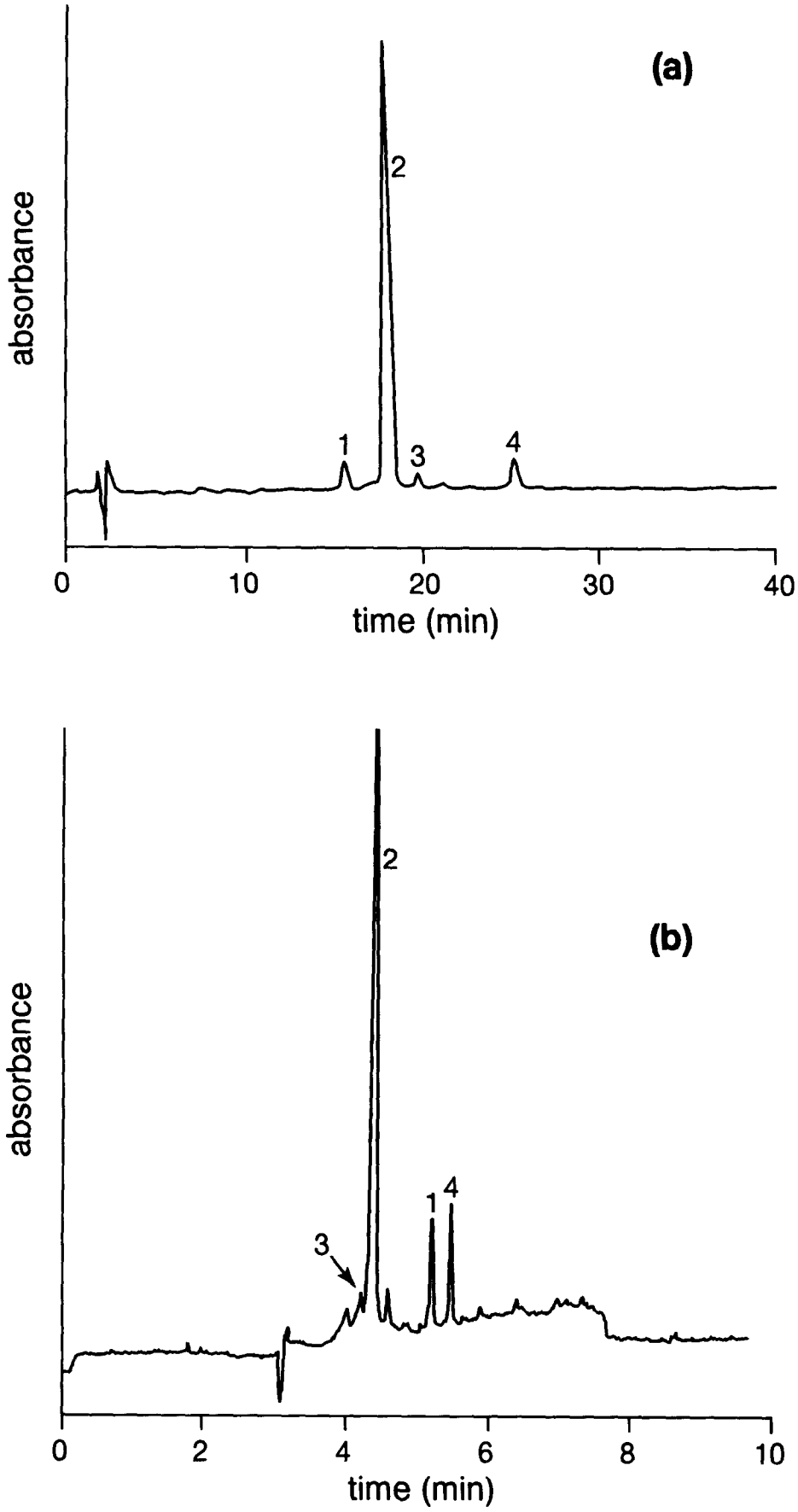

Fig. 1. Strawberry anthocyanins separated by (a) HPLC, (b) CZE. Peak labels: 1—cyanidin 3-glucoside, 2—pelargonidin 3-glucoside, 3—pelargonidin 3-rutinoside, 4—pelargonidin 3-succinylglucoside.

The elderberry anthocyanins showed good separation (Fig. 2a); the 3,5-diglycosides eluted first (cyanidin 3-sambubioside-5-glucoside—I (main pigment) and cyanidin 3,5-diglucoside—II), followed by the 3-glycosides (cyanidin 3-sambubioside—III and cyanidin 3-glucoside—IV).

CZE

Separation by CZE is based on a different selectivity process than that for HPLC. We have discussed factors influencing the order of elution of anthocyanins in CZE elsewhere (Bridle *et al.*, 1996), but briefly, the

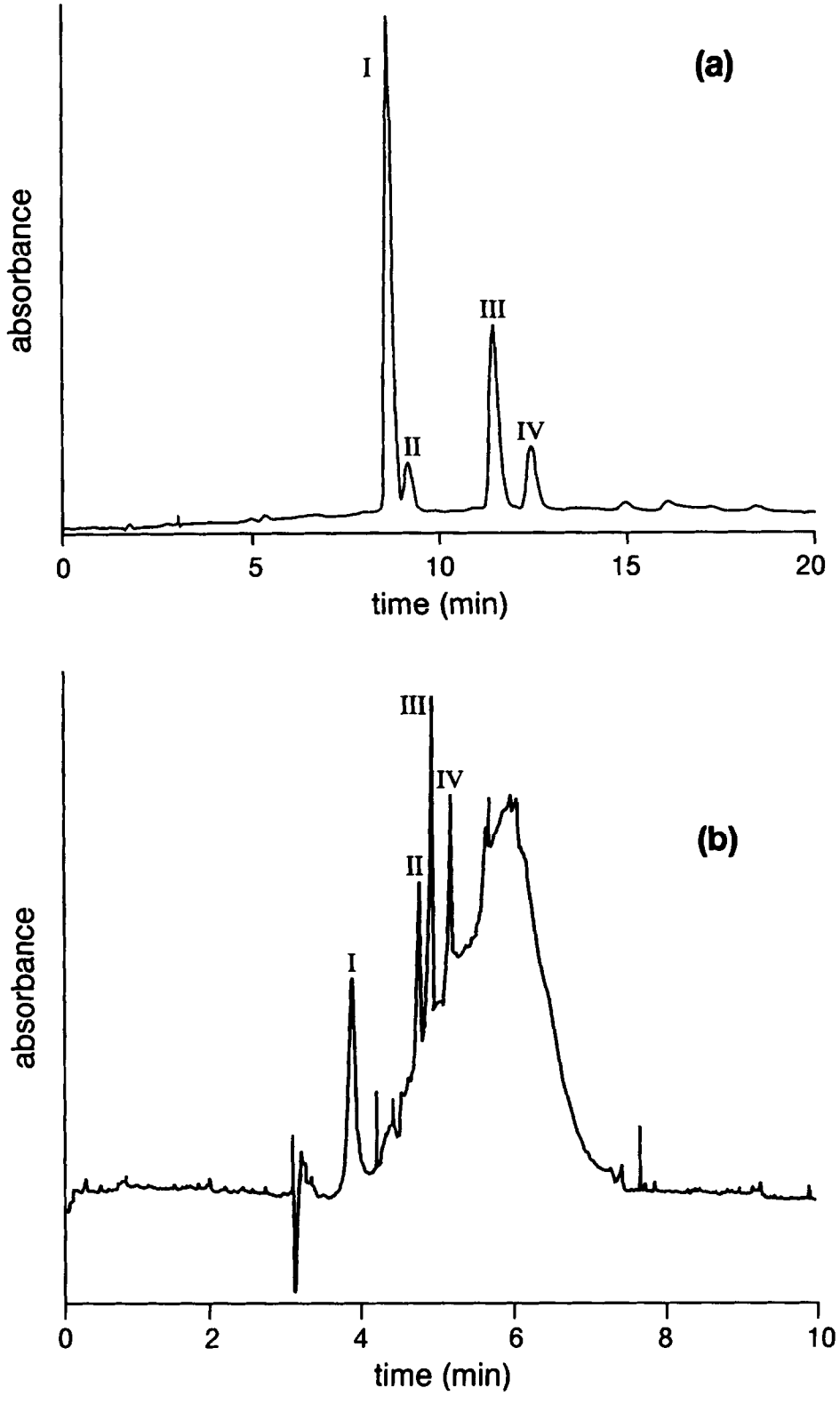

Fig. 2. Elderberry anthocyanins separated by (a) HPLC, (b) CZE. Peak labels: I—cyanidin 3-sambubioside-5-glucoside, II—cyanidin 3,5-diglucoside, III—cyanidin 3-sambubioside, IV—cyanidin 3-glucoside.

electro-osmotic flow is the main driving force under our conditions, while increased glycosylation shortens migration time and anthocyanins having greater negative charge densities have reduced electrophoretic mobility; the effect of complex formation between borate in the buffer and anthocyanins having ortho-dihydroxylation is an additional factor influencing mobility. Thus, in the strawberry extract (Fig. 1b), pelargonidin 3-rutinoside (peak 3) eluted before the main anthocyanin (pelargonidin 3-glucoside—peak 2), followed by cyanidin 3-glucoside (1) and pelargonidin 3-succinylglucoside (4). Some small unidentified peaks were also observed, which were noted earlier (Bakker *et al.*, 1994).

The electropherogram of elderberry anthocyanins was less satisfactory owing to the large baseline hump underlying the anthocyanin peaks (Fig. 2b). However, it is still possible to observe the individual anthocyanins in the mixture, which nevertheless, were better resolved by CZE than by HPLC. The elution order was the same as by HPLC (cyanidin 3-sambubioside-5-glucoside (main)—I, cyanidin 3,5-diglucoside—II, cyanidin 3-sambubioside—III and cyanidin 3-glucoside—IV). The cause of the rising baseline in this analysis is possibly due to additional material (coloured polymers) extracted by the acidified methanolic solvent, contrasting with the milder juicing process used to obtain the strawberry sample. The elderberry extract also contained a high concentration of non-coloured compounds absorbing at 280 nm (HPLC—not shown), which may also exert an adverse effect on the baseline and on subsequent quantitation. Both techniques are capable of providing reliable quantitative analytical information, and we have shown such data elsewhere for the analysis of non-coloured phenolic compounds (García-Viguera & Bridle, 1995). Because of the varying requirements of each method in this present comparison, it is sufficient to record here the relative proportions of anthocyanins expressed as normalised area per cent in both mixtures by both methods (Table 1). These results show good agreement between the methods for the strawberry sample, but are less satisfactory for the elderberry extract, for reasons mentioned above relating to the rising baseline.

Unlike other flavonoid compounds, anthocyanins exist in a pH dependent equilibrium (Brouillard, 1982), with the principal coloured (flavylium) species dominant only below pH 2. Hence most existing chromatographic separation techniques use acidic conditions for the selective detection of the coloured flavylium species, either by eye, or at a visible wavelength which precludes non-coloured components from appearing as peaks in the chromatogram. However, most capillary electrophoresis separations have been developed using capillaries suitable for use with neutral pH buffers, in which pH range, the anthocyanins are coloured only weakly by a very small amount of the quinonoidal base form, the dominant species being other colourless forms. An example of the influence of pH on the anthocyanin absorbance spectrum is illustrated for pelargonidin 3-glucoside in Fig. 3, albeit with differing concentrations of pigment.

Table 1. Relative amounts of each peak according to analytical method

Strawberry:	Peak	1	2	3	4
HPLC		6·4	85·5	2·9	5·2
CZE		6·8	83·9	2·2	7·1
Elderberry:	Peak	I	II	III	IV
HPLC		55·5	8·1	26·0	10·4
CZE		42·6	15·9	27·0	14·5

Peaks, expressed as area per cent (CZE normalised peak areas), are mean values of duplicate determinations.

It has been stressed that CZE demands high analyte concentration in a small volume (Tomás-Barberán, 1995). This observation is compounded by several features in this present work. Thus, a concentrated CZE analysis sample is required for adequate detection of sample components for reasons of anthocyanin chemistry described above. Further factors are the differences in the parameters of the systems, viz. the sample volumes—9 nL CZE; 25 μL HPLC, (the minimal introduction volume in CZE is a direct consequence of the small capillary diameter) and detector path lengths (75 μm CZE; 6 mm HPLC). The detection limit of each method was compared using the strawberry anthocyanin mixture. For HPLC, 0·046 mg mL^{-1} (0·11 mM expressed as pelargonidin 3-glucoside) was the minimum concentration for detection of the minor peaks, whereas 4·0 mg/mL (9·6 mM) was required for CZE. This approximates to an 87 times greater concentration requirement for CZE than HPLC.

On a comparative basis, CZE lacks the flexibility afforded by the ability to use solvent gradients in HPLC separations, but it has the potential to optimise separations by varying other parameters (e.g. temperature, voltage, electrolyte concentration) and also the use of additives (complexing agents, organic modifiers, micelles). CZE has minimal set-up time, low running costs and can give better separation efficiencies in a shorter run time, while HPLC is more rugged and has an established versatility; it is also easily adaptable for preparative work. The need in CZE for highly concentrated samples is a drawback when precious samples are in short supply, but the prospects for the analysis of this important group of natural colourants may be significantly enhanced, if methodology for CZE under strongly acidic conditions becomes available.

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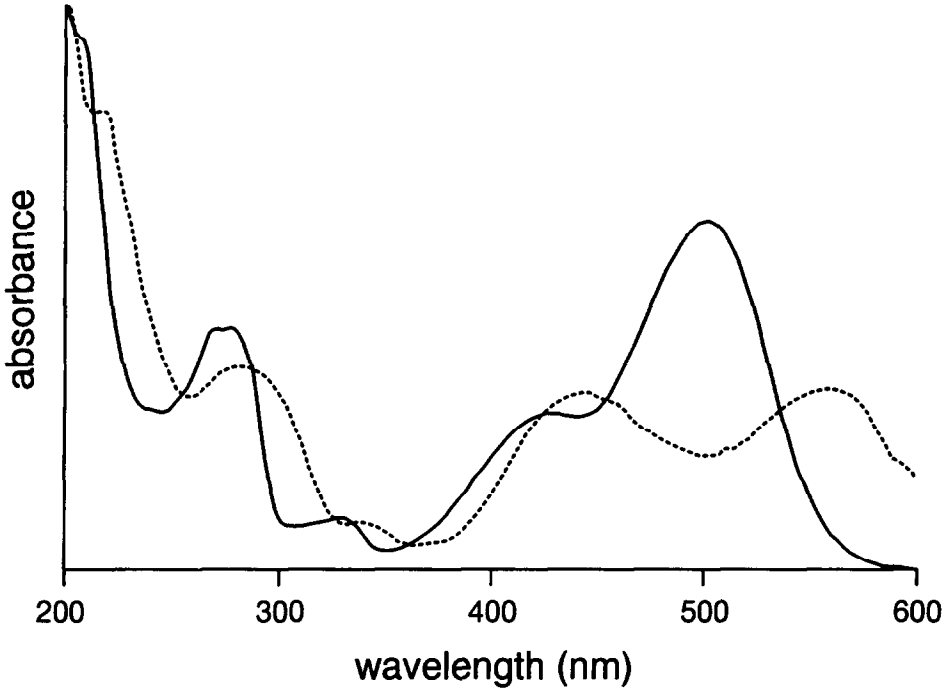

Fig. 3. Diode array detector spectra of pelargonidin 3-glucoside compared at pH 1·8 (HPLC—solid line) and pH 8.0 (CZE—dotted line, at 87 times greater sample concentration). DAD sampling interval; HPLC: 1280 ms, spectrum in methanol (28%)—0·6% $HClO_4$ (72%); CZE: 160 ms, spectrum in borate buffer.

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