

Capillary Electrophoresis Equipped with Laser Induced Fluorescence: A Novel Technique for Separation and Identification of Natural Dyes from Historical Textiles

By

Shokoufeh Ahmadi

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Department of Chemistry
Faculty of Science
University of Manitoba
Winnipeg, MB, Canada

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Abstract

The aim of this research was to evaluate the feasibility of capillary electrophoresis in micellar electrokinetic mode (MEKC) equipped with laser induced fluorescence (LIF) as a novel technique to study dyes extracted from valuable textiles. CE identification of dyes requires extraction of dyes from the substrate, which is one of the most challenging aspects in conservation science. Therefore, developing a technique with minimum destructive effect on objects is favorable. The most important advantages of using LIF are the low detection limits and high sensitivity which allow for the separation of very small quantities of sample, which makes it suitable for working with historical objects.

Feasibility studies were performed to explore separation properties of a small number of historical dyes (alizarin, purpurin, carmine and morin) using: different concentrations of modifiers and borate buffer at different pH values, and applied voltages. By manipulating separation parameters the optimum separation condition for a mixture of 10 dyes was defined as: under 22 kV, 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile. The separation of flavonols and purpurin using CE at pH>9.2 appeared to be complicated by reproducibility in peak height and peak shape of purpurin. In this study the poor peak reproducibility of CE separations was attributed to photochemical properties of dyes.

The 1st order rate constants (k) for the decomposition of purpurin and selected flavonol dyes were determined under different pH conditions, k for purpurin at pH=9.0 under dark condition was $9 \times 10^{-6} s^{-1}$. The stability of these dyes was greatly affected by pH, a modest decrease in pH (pH = 8.5) improved the stability and peak reproducibility of these dyes. To extract dyes from textiles, the existing extraction methods with focus on Hansen solubility parameters were evaluated. It appeared that methanol was the most suitable solvent

and the dyes in the following samples were extracted by methanol and identified running CE-LIF: textiles from “Salt Man” (600 BC), Safavid dynasty and Chinese robe (1700 AD). CE-LIF was used successfully for separation and identification of small quantities of natural dyes extracted from less than 1 mg samples such as wool, silk and paper.

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Dedication

Thanks to my family who nurtured me with great love.

This thesis is dedicated to the memory of my beloved father, Mehdi Ahmadi, who is always in my heart. Dad, you are deeply appreciated for your constant pledge to my graduation pursuit. I hope you are resting in peace by making your last wish come true.

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Figure 3.11 Separation of selected anthraquinones and flavonoid dyes (3 $\mu\text{g ml}^{-1}$ alizarin, 3 $\mu\text{g ml}^{-1}$ purpurin, 1 $\mu\text{g ml}^{-1}$ emodin, 2 $\mu\text{g ml}^{-1}$ myricetin, 10 $\mu\text{g ml}^{-1}$ carmine, 1 $\mu\text{g ml}^{-1}$ quercetin, 0.5 $\mu\text{g ml}^{-1}$ morin, 2 $\mu\text{g ml}^{-1}$ luteolin, 0.5 $\mu\text{g ml}^{-1}$ kaempferol and 1 $\mu\text{g ml}^{-1}$ apigenin in a 20 mM borate buffer and 20 mM SDS at pH= 8.0: **(a)** no modifier, **(b)** in 5% (v/v), and **(c)** in 10% (v/v) of acetonitrile at 18 kV. The assigned peaks for **(a)** and **(b)** are: (1) alizarin, (2) purpurin, (3) emodin, (4) myricetin and carmine, (5) quercetin and morin, (6) luteolin, (7) kaempferol, and (8) apigenin for **(c)** are the same as in (a) and (b), except for (3) emodin and myricetin, (4) carmine. 121

Figure 3.12 Separation of selected anthraquinones and flavonoid dyes (3 $\mu\text{g ml}^{-1}$ alizarin, 3 $\mu\text{g ml}^{-1}$ purpurin, 1 $\mu\text{g ml}^{-1}$ emodin, 2 $\mu\text{g ml}^{-1}$ myricetin, 10 $\mu\text{g ml}^{-1}$ carmine, 1 $\mu\text{g ml}^{-1}$ quercetin, 0.5 $\mu\text{g ml}^{-1}$ morin, 2 $\mu\text{g ml}^{-1}$ luteolin, 0.5 $\mu\text{g ml}^{-1}$ kaempferol) in a 20 mM borate buffer and 20 mM SDS at pH= 8.0: **(a)** no modifier, **(b)** in 5% (v/v), and **(c)** in 10% (v/v) of methanol at 18 kV. The assigned peaks for **(a), (b)** and **(c)** are: (1) alizarin,

(2) purpurin, (3) emodin, (4) myricetin and carmine, (5) quercetin and morin, (6) luteolin, (7) kaempferol, and (8) apigenin. 123

Figure 3.13 Separation of selected anthraquinones and flavonoid dyes ($3 \mu\text{g ml}^{-1}$ alizarin, $3 \mu\text{g ml}^{-1}$ purpurin, $1 \mu\text{g ml}^{-1}$ emodin, $2 \mu\text{g ml}^{-1}$ myricetin, $10 \mu\text{g ml}^{-1}$ carmine, $1 \mu\text{g ml}^{-1}$ quercetin, $0.5 \mu\text{g ml}^{-1}$ morin, $2 \mu\text{g ml}^{-1}$ luteolin, $0.5 \mu\text{g ml}^{-1}$ kaempferol) in a 20 mM borate buffer and 20 mM SDS at pH= 8.0, at: (a) 18 kV, (b) 20 kV, (c) 22 kV and (d) 25 kV. The assigned peaks for (a), (b) and (d) are: (1) alizarin, (2) purpurin, (3) emodin, (4) carmine, (5) myricetin, (6) quercetin and morin, (7) luteolin, (8) the impurity from emodin, (9) kaempferol, and (10) apigenin. In run (c) the peaks (1 to 7) are the same as assigned in other runs except peak (8) is kaempferol, and (9) is apigenin. Reproduced with permission from Ref [49]. – Copyright: MDPI AG..... 124

Figure 3.14 Separation of selected anthraquinones and flavonoid dyes (as mentioned in Figure 3.13) in presence 5% (v/v) of acetonitrile in a 20 mM borate buffer and 20 mM SDS at pH= 8.0, at: (a) 18kV, (b) 20 kV, (c) 22 kV and (d) 25 kV. The assigned peaks for (a), (b) and (d) are: (1) alizarin, (2) purpurin, (3) emodin, (4) carmine, (5) myricetin, (6) quercetin and morin, (7) luteolin, (8) the impurity from emodin, (9) kaempferol, and (10) apigenin. In run (c) the peaks are the same as assigned in other runs except peak (8) is kaempferol, and (9) is apigenin. (d:) Adapted from Ref. [26] – Copyright: Elsevier 125

Figure 3.15 Separation of selected anthraquinones and flavonoid dyes (as mentioned in Figure 3.13) in presence 5% (v/v) of methanol in a 20 mM borate buffer and 20 mM SDS at pH= 8.0: (a) 18kV, (b) 20 kV, (c) 22 kV and (d) 25 kV. The assigned peaks for (a): (1) alizarin, (2) purpurin, (3) emodin, (4) carmine + myricetin, (5) quercetin and morin, (6) luteolin, (7) kaempferol, and (8) apigenin. In (b) the peaks are: (1) alizarin, (2) purpurin, (3) emodin, (4) carmine (5) myricetin, (6) quercetin, (7) morin, (8) luteolin, (9) kaempferol, and (10) apigenin. In runs (c) and (d) the peaks assigned as: (1) alizarin, (2) purpurin, (3) emodin, (4) carmine, (5) myricetin, (6) quercetin and morin, (7) luteolin, (8) kaempferol, and (9) apigenin. 125

Figure 3.16 Separation of selected anthraquinones and flavonoid dyes in a 20 mM borate buffer and 20 mM SDS at pH= 8.0 using 20 kV. In run (a) peak identities: (1) alizarin, (2) purpurin, (3) myricetin, (4) and morin. Run (b) the peaks are: (1) alizarin, (2) purpurin, (3) myricetin, (4) quercetin + morin. Run (c) the peaks are assigned as: (1) alizarin, (2) purpurin, (3) carmine, (4) myricetin, (5) quercetin + morin. 126

Figure 3.17 Separation of anthraquinones and flavonoid dyes in a 20 mM borate buffer and 20 mM SDS at pH= 8.0 using an applied potential of 25 kV. The concentrations of dyes were: $3 \mu\text{g ml}^{-1}$ alizarin, $3 \mu\text{g ml}^{-1}$ purpurin, $1 \mu\text{g ml}^{-1}$ emodin, $2 \mu\text{g ml}^{-1}$ myricetin, $10 \mu\text{g ml}^{-1}$ carmine, $1 \mu\text{g ml}^{-1}$ quercetin, $0.5 \mu\text{g ml}^{-1}$ morin, $2 \mu\text{g ml}^{-1}$ luteolin, $0.5 \mu\text{g ml}^{-1}$ kaempferol, and $1 \mu\text{g ml}^{-1}$ apigenin. Peak identities: run (a): (1) alizarin, (2) purpurin, (3) emodin, (4) myricetin and carmine, (5) quercetin and morin, (6) luteolin, (7) the impurity from emodin, (8) kaempferol, and (9) apigenin. Run (b): (1) alizarin, (2) purpurin, (3) emodin, (4) myricetin and carmine, (5) morin, (6) the impurity from emodin, (7) luteolin, (8) kaempferol, and (9) apigenin. Run (c): (1) alizarin, (2) purpurin, (3) myricetin and carmine, (4) quercetin and morin, (5) kaempferol, and (6) apigenin. Run (d): (1) alizarin, (2) purpurin, (3) carmine, (4) quercetin and morin, (5) luteolin, (6) kaempferol, and (7) apigenin. 128

Figure 3.18 Separation of selected anthraquinones and flavonoid dyes ($1 \mu\text{g ml}^{-1}$ alizarin, $3 \mu\text{g ml}^{-1}$ purpurin, $1 \mu\text{g ml}^{-1}$ emodin, $2 \mu\text{g ml}^{-1}$ myricetin, $1 \mu\text{g ml}^{-1}$ quercetin, $0.5 \mu\text{g ml}^{-1}$

morin, 2 $\mu\text{g ml}^{-1}$ luteolin, 0.5 $\mu\text{g ml}^{-1}$ kaempferol, 1 $\mu\text{g ml}^{-1}$ apigenin,) in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, (a) no modifier, (b) in 5% (v/v), and (c) in 10% (v/v) of acetonitrile at 18 kV. The assigned peaks for run **(a)**: (1) alizarin, (2) emodin, (3) purpurin, (4) Kaempferol, (5) apigenin/ myricetin, (6) luteolin/ quercetin and (7) morin. Run **(b)**: (1) alizarin, (2) emodin, (3) purpurin, (4) Kaempferol, (5) myricetin, (6) apigenin, (7) luteolin, (8) quercetin and (9) morin. Run **(c)**: (1) alizarin, (2) emodin, (3) purpurin, (4) Kaempferol, (5) apigenin, (6) myricetin, (7) luteolin, (8) quercetin and (9) morin. 133

Figure 3.19 Separation of selected anthraquinones and flavonoid dyes (3 $\mu\text{g ml}^{-1}$ alizarin, 3 $\mu\text{g ml}^{-1}$ purpurin, 1 $\mu\text{g ml}^{-1}$ emodin, 2 $\mu\text{g ml}^{-1}$ myricetin, 10 $\mu\text{g ml}^{-1}$ carmine, 1 $\mu\text{g ml}^{-1}$ quercetin, 0.5 $\mu\text{g ml}^{-1}$ morin, 2 $\mu\text{g ml}^{-1}$ luteolin, 0.5 $\mu\text{g ml}^{-1}$ kaempferol, 1 $\mu\text{g ml}^{-1}$ apigenin,) in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, run **(a)** no modifier, **(b)** in 5% (v/v), and **(c)** in 10% (v/v) of methanol at 18 kV. The assigned peaks for run **(a)**: (1) alizarin, (2) purpurin, (3) Kaempferol, (4) emodin, (5) apigenin/ myricetin/ carmine, (6) luteolin/quercetin and (7) morin. Run **(b)**: (1) alizarin, (2) purpurin, (3) Kaempferol/ emodin, (4) apigenin/ myricetin/carmine, (5) luteolin/ quercetin and (6) morin. Run **(c)**: (1) alizarin, (2) purpurin, (3) Kaempferol, (4) emodin, (5) apigenin, (6) carmine, (7) myricetin, (8) luteolin, (9) quercetin and (10) morin. 135

Figure 3.20 Separation of selected anthraquinones and flavonoid dyes (the same concentration used in Figure 3.19) in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, at: **(a)** 18 kV, **(b)** 20 kV, **(c)** 22 kV and **(d)** 25 kV. The assigned peaks for **(a)**: (1) alizarin, (2) purpurin, (3) kaempferol, (4) emodin, (5) myricetin, (6) apigenin/ carmine, (7) luteolin, (8) quercetin, (9) morin. Run **(b)**: (1) alizarin, (2) purpurin, (3) kaempferol, (4) emodin, (5) apigenin, (6) carmine, (7) myricetin, (8) luteolin, (9) quercetin, and (10) morin. Run **(c)**: (1) alizarin, (2) purpurin, (3) kaempferol, (4) emodin, (5) apigenin/ myricetin, (6) carmine, (7) luteolin/ quercetin, (8) morin. Run **(d)**: (1) alizarin, (2) purpurin, (3) kaempferol, (4) emodin, (5) myricetin, (6) apigenin/ carmine, (7) luteolin (8) quercetin, and (9) morin. 136

Figure 3.21 Separation of selected anthraquinones and flavonoid dyes (as mentioned in Figure 3.19) in presence 5% (v/v) of acetonitrile in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, at: **(a)** 18 kV, **(b)** 20 kV **(c)** 22 kV and **(d)** 25 kV. The assigned peaks for **(a)** and **(b)** : (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin, (6) carmine, (7) myricetin, (8) luteolin, (9) quercetin, (10) morin. Run **(c)** and **(d)**: (1) alizarin, (2) purpurin, (3) kaempferol, (4) emodin, the rest is the same as in run (a). 137

Figure 3.22 Separation of selected anthraquinones and flavonoid dyes (as mentioned in Figure 3.19) in presence 5% (v/v) of methanol in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, at: **(a)** 18 kV, **(b)** 20 kV **(c)** 22 kV and **(d)** 25 kV. The assigned peaks for run **(a)** and **(d)**: (1) alizarin, (2) purpurin, (3) kaempferol, (4) emodin, (5) apigenin/carmine (6) myricetin, (7) luteolin, (8) quercetin, (9) morin. Run **(b)** and **(c)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin, (6) carmine, (7) myricetin, (8) luteolin, (9) quercetin, (10) morin. 138

Figure 3.23 Separation of a mixture of dyes in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, in 5% (v/v) of methanol, using an applied potential of 25 kV. The concentrations of dyes were: 3 $\mu\text{g ml}^{-1}$ alizarin, 3 $\mu\text{g ml}^{-1}$ purpurin, 5 $\mu\text{g ml}^{-1}$ emodin, 2 $\mu\text{g ml}^{-1}$ myricetin, 10 $\mu\text{g ml}^{-1}$ carmine, 2 $\mu\text{g ml}^{-1}$ quercetin, 0.2 $\mu\text{g ml}^{-1}$ morin. Peak identities: run **(a)**: (1) alizarin, (2) purpurin, (3) carmine, (4) morin. Run **(b)**: (1) alizarin,

(2) purpurin, (3) emodin, (4) carmine, (5) morin. Run **(c)**: (1) alizarin, (2) purpurin, (3) emodin, (4) carmine, (5) myricetin, (6) morin. Run **(d)**: (1) alizarin, (2) purpurin, (3) emodin, (4) carmine, (5) myricetin/ quercetin, (6) morin. 139

Figure 3.24 Separation of a mixture of dyes in a 20 mM borate buffer and 20 mM SDS at pH= 8.5 using an applied potential of 18 kV. Peak identities: run **(a)**: (1) 3 $\mu\text{g ml}^{-1}$ alizarin, (2) 3 $\mu\text{g ml}^{-1}$ purpurin, (3) 0.5 $\mu\text{g ml}^{-1}$ morin. Run **(b)**: (1) 3 $\mu\text{g ml}^{-1}$ alizarin, (2) 3 $\mu\text{g ml}^{-1}$ purpurin, (3) 0.5 $\mu\text{g ml}^{-1}$ morin, (4) 1 $\mu\text{g ml}^{-1}$ emodin, (5). Run **(c)**: (1) 5 $\mu\text{g ml}^{-1}$ alizarin, (2) 3 $\mu\text{g ml}^{-1}$ purpurin, (3) 5 $\mu\text{g ml}^{-1}$ emodin, (5) 0.5 $\mu\text{g ml}^{-1}$ morin. Run **(d)**: (1) 10 $\mu\text{g ml}^{-1}$ alizarin, (2) 3 $\mu\text{g ml}^{-1}$ purpurin, (3) 0.5 $\mu\text{g ml}^{-1}$ morin, (4) 5 $\mu\text{g ml}^{-1}$ emodin. Run **(e)**: (1) 5 $\mu\text{g ml}^{-1}$ alizarin and 10 $\mu\text{g ml}^{-1}$ purpurin, (2) $\mu\text{g ml}^{-1}$ morin, (3) 10 $\mu\text{g ml}^{-1}$ emodin. 140

Figure 3.25 Separation of a mixture of dyes in a 20 mM borate buffer and 20 mM SDS at pH= 8.5 using an applied potential of 18 kV. Peak identities: Run **(a)**: (1) 0.5 $\mu\text{g ml}^{-1}$ kaempferol and 1 $\mu\text{g ml}^{-1}$ emodin, (2) 1 $\mu\text{g ml}^{-1}$ apigenin and (3) 2 $\mu\text{g ml}^{-1}$ myricetin, 1 $\mu\text{g ml}^{-1}$ quercetin and 2 $\mu\text{g ml}^{-1}$ luteolin. **(b)**: 2 $\mu\text{g ml}^{-1}$ myricetin, **(c)**: 1 $\mu\text{g ml}^{-1}$ quercetin, **(d)**: 1 $\mu\text{g ml}^{-1}$ apigenin, **(e)**: 1 $\mu\text{g ml}^{-1}$ emodin, **(f)**: 0.5 $\mu\text{g ml}^{-1}$ kaempferol, and **(g)**: 2 $\mu\text{g ml}^{-1}$ luteolin. 141

Figure 3.26 Separation of a mixture of flavonoids in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, in 5% (v/v) of acetonitrile, using an applied potential of 22 kV. Peak identities: Run **(a)**: (1) 2 $\mu\text{g ml}^{-1}$ lu-7-*O*-glu, (2) 2 $\mu\text{g ml}^{-1}$ ap-7-*O*-glu, (3) 2 $\mu\text{g ml}^{-1}$ kaempferol, (4) 2 $\mu\text{g ml}^{-1}$ apigenin, 6 $\mu\text{g ml}^{-1}$ myricetin, (5) 2 $\mu\text{g ml}^{-1}$ luteolin, (6) 1 $\mu\text{g ml}^{-1}$ quercetin, (7) 1 $\mu\text{g ml}^{-1}$ morin. Run **(b)**: (1) 2 $\mu\text{g ml}^{-1}$ lu-7-*O*-glu, (2) 2 $\mu\text{g ml}^{-1}$ ap-7-*O*-glu (3) 1 $\mu\text{g ml}^{-1}$ kaempferol, (4) 4 $\mu\text{g ml}^{-1}$ apigenin, 6 $\mu\text{g ml}^{-1}$ myricetin, (4) 1 $\mu\text{g ml}^{-1}$ luteolin, (6) 2 $\mu\text{g ml}^{-1}$ quercetin, (7) 1 $\mu\text{g ml}^{-1}$ morin. 143

Figure 3.27 Separation of a mixture of flavonoids in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, in 5% (v/v) of acetonitrile, using an applied potential of 22 kV. Peak identities: (1) 2 $\mu\text{g ml}^{-1}$ lu-7-*O*-glu, (2) 2 $\mu\text{g ml}^{-1}$ ap-7-*O*-glu, (3) 1 $\mu\text{g ml}^{-1}$ kaempferol, (4) 1 $\mu\text{g ml}^{-1}$ apigenin, (5) 1 $\mu\text{g ml}^{-1}$ luteolin/ 1 $\mu\text{g ml}^{-1}$ quercetin, (6) 1 $\mu\text{g ml}^{-1}$ morin. 144

Figure 3.28 Separation of selected anthraquinones and flavonoid dyes (5 $\mu\text{g ml}^{-1}$ alizarin, 10 $\mu\text{g ml}^{-1}$ purpurin, 1 $\mu\text{g ml}^{-1}$ emodin, 0.5 $\mu\text{g ml}^{-1}$ kaempferol, 1 $\mu\text{g ml}^{-1}$ apigenin, 2 $\mu\text{g ml}^{-1}$ luteolin, 10 $\mu\text{g ml}^{-1}$ carmine, 2 $\mu\text{g ml}^{-1}$ myricetin, 1 $\mu\text{g ml}^{-1}$ quercetin, 0.5 $\mu\text{g ml}^{-1}$ morin) , in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, **(a)** no modifier, **(b)** in 5% (v/v), and **(c)** in 10% (v/v) of acetonitrile at 20 kV. The assigned peaks for **(a)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin/luteolin, (6) carmine, (7) myricetin, (8) quercetin and (9) morin. Run **(b)** and **(c)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin, (6) luteolin, (7) carmine, (8) myricetin, (9) quercetin and (10) morin. 145

Figure 3.29 A close up of run b and c in Figure 3.28 146

Figure 3.30 Separation of selected anthraquinones and flavonoid dyes (5 $\mu\text{g ml}^{-1}$ alizarin, 10 $\mu\text{g ml}^{-1}$ purpurin, 10 $\mu\text{g ml}^{-1}$ emodin, 0.5 $\mu\text{g ml}^{-1}$ kaempferol, 1 $\mu\text{g ml}^{-1}$ apigenin, 2 $\mu\text{g ml}^{-1}$ luteolin, 2 $\mu\text{g ml}^{-1}$ myricetin, 1 $\mu\text{g ml}^{-1}$ quercetin, 0.5 $\mu\text{g ml}^{-1}$ morin), in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, **(a)** no modifier, **(b)** in 5% (v/v), and **(c)** in 10% (v/v) of methanol at 20 kV. The assigned peaks for **(a)**: (1) alizarin, (2) emodin, (3) purpurin, (4) kaempferol, (5) apigenin/luteolin, (6) myricetin, (7) quercetin and (8) morin. Run **(b)**: (1) alizarin, (2) emodin, (3) purpurin, (4) kaempferol, (5) apigenin, (6) luteolin,

(7) myricetin, (8) quercetin and (9) morin; run **(c)** (1) alizarin, (2) emodin, (3) purpurin, (4) kaempferol, (5) apigenin, (6) luteolin/ myricetin, (7) quercetin and (8) morin. **147**

Figure 3.31 Separation of selected anthraquinones and flavonoid dyes ($10\ \mu\text{g ml}^{-1}$ alizarin, $10\ \mu\text{g ml}^{-1}$ purpurin, $1\ \mu\text{g ml}^{-1}$ emodin, $0.5\ \mu\text{g ml}^{-1}$ kaempferol, $1\ \mu\text{g ml}^{-1}$ apigenin, $2\ \mu\text{g ml}^{-1}$ luteolin, $10\ \mu\text{g ml}^{-1}$ carmine, $2\ \mu\text{g ml}^{-1}$ myricetin, $1\ \mu\text{g ml}^{-1}$ quercetin, $0.5\ \mu\text{g ml}^{-1}$ morin), in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, **(a)** no modifier, **(b)** in 5% (v/v), and **(c)** in 10% (v/v) of methanol at 20 kV. The assigned peaks for **(a)** and **(c)**: (1) alizarin, (2) emodin, (3) purpurin, (4) kaempferol, (5) apigenin/luteolin, (6) carmine, (7) myricetin, (8) quercetin and (9) morin. Run **(b)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) luteolin, (6) apigenin/carmine, (7) myricetin, (8) quercetin and (9) morin. **148**

Figure 3.32 Separation of selected anthraquinones and flavonoid dyes ($10\ \mu\text{g ml}^{-1}$ alizarin, $3\ \mu\text{g ml}^{-1}$ purpurin, $1\ \mu\text{g ml}^{-1}$ emodin, $0.5\ \mu\text{g ml}^{-1}$ kaempferol, $1\ \mu\text{g ml}^{-1}$ apigenin, $2\ \mu\text{g ml}^{-1}$ luteolin, , $10\ \mu\text{g ml}^{-1}$ carmine, $2\ \mu\text{g ml}^{-1}$ myricetin, $1\ \mu\text{g ml}^{-1}$ quercetin, $0.5\ \mu\text{g ml}^{-1}$ morin) , in a 20 mM borate buffer and 20 mM SDS at pH= 9.2 , at: **(a)** 18 kV, **(b)** 20 kV, **(c)** 22 kV and **(d)** 25 kV The assigned peaks for **(a)** : (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin, (6) luteolin, (7) carmine, (8) myricetin, (9) quercetin and (10) morin. Run **(b)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin/ luteolin/ carmine, (6) myricetin, (7) quercetin and (8) morin. Run **(c)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) luteolin, (6) apigenin/ carmine, (7) myricetin, (8) quercetin and (9) morin. Run **(d)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) luteolin, (6) apigenin, (7) carmine, (8) myricetin, (9) quercetin and (10) morin. Reproduced with permission from Ref [49]. – Copyright: MDPI AG **149**

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List of Symbols

ACN	Acetonitrile
al	alizarin
ap	apigenin
ap-7- <i>O</i> -glu	apigenin-7- <i>O</i> -glucoside
<i>V</i>	Applied voltage
BGE	Background Electrolyte
br	brazilein
CE	Capillary Electrophoresis
CGE	Capillary gel electrophoresis
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachopheresis
CZE	Capillary zone electrophoresis
ca	carminic acid
ear	carthamin
CTAB	Cetyltrimethyl ammonium bromide
chr	chrysoeriol
psp-mu	co elution of pseudo purpurin and munjistin
σ	Conductivity
CMC	Critical Micelle Concentration
cr	crocetin
<i>A</i>	Cross sectional of capillary
<i>i</i>	Current
DCM	dichloromethane
DMSO	Dimethyl sulfoxide
DMF	Dimethylformamide
DAD	Diode array detector
W	Distilled water
<i>E</i>	Electric field strength
EOF	Electroosmotic Flow

μ_{eo}	Electroosmotic mobility
v_{eo}	Electroosmotic velocity
μ_{ep}	Electrophoretic Mobility
v_{ep}	Electrophoretic Velocity
em	emodin
EtOH	Ethanol
FORS	Fiber optic reflectance spectroscopy
FOFS	Fiber-optic fluorescence spectroscopy
fka	flavokermesic acid
FE	Fluorescence Emission
FS	Fluorescence spectroscopy
f	Friction factor
ga	gambogic acid
GC	Gas chromatography
J	Generated heat
gn	genistein
gn- <i>O</i> -gl	genistein glucoside
HPLC	High performance liquid chromatography
ISC	Intersystem crossing
in	indigo
ind	indirubin
IR	Infrared spectroscopy
IC	Internal conversion
r	Ionic radius of the ions
IPA	Isopropanol
kf	kaempferol
Kf-3,7-di-g	kaempferol-3 ,7-diglucoside
ka	kermesic acid
la	laccaic acid
LC	Liquid chromatography
lu	luteolin
lu-7- <i>O</i> -glu	luteolin-7- <i>O</i> -glucoside

MS	Mass spectrometry
ϵ	Medium dielectric constant
MeOH	Methanol
MECC	Micellar electrokinetic capillary chromatography
MEKC-LIF	Micellar Electrokinetic Capillary Chromatography - Laser Induced Fluorescence
mo	morin
mu	munjistin
my	myricetin
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
N	Number of theoretical plates
N.A	Numerical Aperture
σ^2	Peak broadening or peak dispersion
PDA	Photo diode array
PMT	Photomultiplier
Brij-35	Polyoxyethyleneglycol dodecyl ether
psp	pseudo purpurin
p	purpurin
qu	quercetin
Qu-4'-O -g	quercetin 4'-glycoside
qz	quinizarin
RC	Resistor–Capacitor
F_f	Retarding frictional force
ru	rubidian
SFC	Sheath Flow Cuvette
SDS	Sodium dodecyl sulfate
STS	Sodium tetradecyl sulphate
Syn Bl	synthetic blue
N_g	The aggregation number
q	The charge on the ions in the sample
F	The force of electrostatic attraction
R_s	The resolution
TLC	Thin layer chromatography

t_m	Time of migration
L_t	Total length of the capillary
UVS	Ultraviolet-visible spectroscopy
η	Viscosity of the solution
XRF	X ray fluorescence
xp	xanthopurpurin
ζ	Zeta potential
5	

Chapter 1

Introduction and background

1.1 Introduction

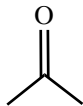
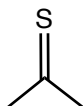
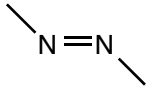
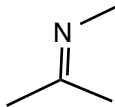
Colour is one of the elements of aesthetics. Johannes Itten believed that the life is colour and the world without colour does not have any feelings and it is dead [1]. The application of colourants dates back 30,000 years ago when people decorated their cave with earth pigments [2].

Colouring agents or colourants are chemical compounds that can be classified as either pigments or dyes. Pigments are inorganic compounds that are suspended in a vehicle; e.g. water and by the help of binder they adhere to a substrate. Oil and gum are common binders to hold the pigments on the canvas surface. Pigments such as yellow ochre, red ochre and soot were applied on the surface of the walls in the caves by mouth spraying or spreading by fingers. The word 'ochre' comes from Greek, which means yellow. Yellow ochre is made of $(\text{Fe}_2\text{O}_3 \cdot \text{H}_2\text{O})$ ferric oxide monohydrate obtained from soil. By heating yellow ochre, it changes to anhydrate form known as red ochre. Also, soot is considered as pigment because it is insoluble in any solvent. Soot was obtained from the burning animal fats or the residue of the burned charcoal [2].

As opposed to pigments which are inorganic compounds, dyes are organic compounds used mostly in dyeing fabrics and in paintings. Dyes can dissolve in a solvent e.g. water and absorb to a substrate (fiber/textile). Natural dyes have been used for centuries extracted from plants, insect's blood or secretions from sea snails. Dyes appear colourful because of the chemical structure which is responsible of inducing colour due to π electrons transitions of chromophores, chromophoric groups or chromogens [3]. The common chromophoric groups are listed in **Table 1.1**. In the following sections, the sources of dyes, classification

of dyes, identification of dyes in historical objects using spectroscopy and separation techniques will be discussed.

Table 1.1 The common chromophoric groups

Name	Chemical structure
vinylene	—CH=CH—
carbonyl	
thiocarbonyl	
nitroso	—NO
nitro	—NO_2
azo	
imino	

1.2 Sources of natural dyes

The dyes with plant origin can be glycones, which are dyes with glucoside groups or aglycones, which are dyes without glucoside group. Dyes can be extracted from different parts of a plant such as: leaves, flowers, fruits, berries, stigmas, roots and barks. The fresh or dried parts are boiled or fermented and then boiled to obtain residue. The residue is considered to be a dye, if it is a glycone dye, it can dissolve in water due to glucoside branch. The best-known plants for dye extraction are: madder (wild madder and Indian madder),

henna, logwood, walnut, pomegranate, fustic, saffron, Persian berries, alkanet, weld, indigo and woad. Dyes can also be extracted from lichens. Examples of these dyes are litmus and archil (orchil) [4, 5].

The dyes with animal origin are obtained from dried bodies of insects, or secretion of molluscs. Kermes, cochineal and lac dye are examples of the dyes from dried bodies while antique purple is a dye from secretion of molluscs [4].

1.3 Classification of dyes

Dyes can be classified based on their colour, application method and chemical groups.

1.3.1 Classification of dyes based on their colour

The dyes can be classified in 3 colours: red, yellow, blue [6], or according to Schweppe 5 groups: red, yellow, blue, orange/brown and black [7].

The best-known historical dyes belonging to red group are madder, cochineal, kermes, lac dye, brazil wood, logwood and Tyrian purple. The dyes belonging to yellow group are weld, Persian berries, old fustic, to blue group are indigo and woad, to orange-brownish group are young fustic, annatto and walnut-shell, and logwood belongs to black group.

1.3.2 Classification of dyes based on their application method

The classification of dyes based on their application methods are as follows: direct, acid (anionic), basic (cationic), mordant, vat, oxidation, disperse and reactive dyes.

Direct dyes attach to the fiber by intermolecular attractions. Ion-dipole, dipole-dipole, and Van der Waals attractions are the examples of intermolecular attractions. The most well-

known example of natural dye belonging to this group is madder, which consists of alizarin and purpurin. The auxochromic group in these dyestuffs is hydroxyl group, which can form hydrogen bonding with OH groups in cellulose [6].

The name of acid dye or anionic dye originated from the application method, since acetic acid or sulfuric acid is added to the dye bath and at the presence of acid the animal fiber (wool, silk) and the dye become positively and negatively charged respectively, and the salt linkage forms between dye and fiber. Most of the dyes with plant and animal origin can be considered as acid dye.

The basic dyes are applied to the animal fibers in alkaline condition and the salt linkage forms between positively charged dye and negatively charged carboxylic group in animal fiber. Among natural dyes, only berberine belongs to this class of dyes.

The dyes with auxochromic groups such as: -OH, -NH₂, -COOH, are suitable to form complex compounds with specific metals. Based on the number of auxochromic groups in the dye it can be mono, di, or poly-functional mordant dye. Mordant dyes show a very good wash-fastness due to the formation of a large complex molecule between dye and fiber. Wash-fastness refers to the resistance of dye to fading or running when washed. Common mordants are mineral salts such as FeSO₄·7H₂O, K₂Cr₂O₇, K₂SO₄·Al₂(SO₄)₃·24H₂O, SnCl₂ and CuSO₄·5H₂O. Mordant dyes give different colours with different metals. For example, madder seems violet, red, bright red, pink and yellow-brown with Fe²⁺, Cr³⁺, Al³⁺, Sn⁴⁺ and Cu²⁺ respectively. It should be noted these colours can be affected by conditions and other chemicals used in dying.

Vat dye is an insoluble dye and cannot be applied on the textiles but it is soluble in its reduced form. Therefore, by reducing agent it is changed to soluble form also known as

leuco and applied on the textiles such as silk and cotton then it is left at room temperature to oxidise and change to its insoluble form. Vat dyes attach to fibers by intermolecular attraction such as van der Waals and dipole-dipole interactions. Therefore, they do not have a good wash-fastness [6]. Examples of natural vat dyes are woad and indigo.

Oxidation dyes are colourless but when they react with oxidants such as the oxides of iron, copper, chromium and manganese, they become colourful. The dyes belong to this group have an aromatic structure with amine functional groups. Aniline black is an example of an oxidation dye.

Disperse dyes are hydrophobic and therefore insoluble in water. They have an affinity to hydrophobic textile such as acetate (synthetic) fibers. Disperse dyes usually have azo chromophoric group. An example of this dye is Disperse Orange 13 (4-[4-(Phenylazo)-1-naphthylazo]phenol).

Reactive dyes consist of 3 parts: chromophoric group, bridge and reactive group. The role of bridge is to attach the other 2 parts to each other. When reactive dye is used, its reactive group reacts with the hydroxyl groups on the substrate to form a covalent bond. Therefore, textiles dyed with reactive dyes have very good wash-fastness. Examples of reactive dyes are the dyes containing azo and anthraquinone chromophoric groups such as reactive blue 4 (1-Amino-4-[3-(4,6-dichlorotriazin-2-ylamino)-4-sulfophenylamino]anthraquinone-2-sulfonic acid) [6].

1.3.3 Classification of dyes based on the chemical groups

Dyes can also be classified based on their chemical structures. In this section some of the representative and historically important natural dyes are introduced.

Anthraquinone dyes

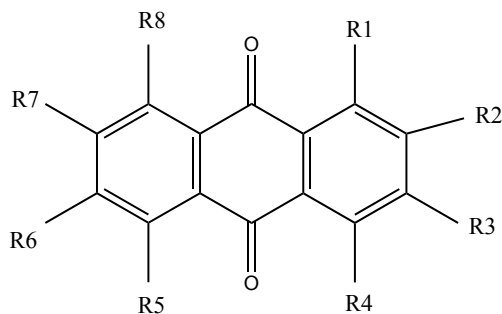
Most of the historically important natural dyes belong to the anthraquinone group. The basic skeleton of the anthraquinone dyes (AQ) is anthraquinone (see **Figure 1.1**). This class of dyes has a good light-fastness which means they resist to fading when exposed to light [4]. The most well-known examples of these dyes are in madder family, rhubarb, Indian berries and red dyes from dried body of animals such as kermes. The dyestuffs in madder (*Rubia tinctorum* L.) and wild madder (*Rubia peregrine* L.) roots are mostly alizarin and purpurin, while Indian madder (*Rubia mungita* L.) mostly contains munjustin [6]. The root of evergreen madder plant is used for dying. This plant has yellow petals and blue to black berries. The quality of madder is related to the soil used for cultivation, the studies showed that the madder grown in chalky soil has better quality for dyeing [8]. Madder is used in dyeing wool, leather, silk and cotton. Madder was one the most popular plants used in Egypt. The linen found in Tutankhamun tomb dating 1823 BC was claimed to be dyed with madder, as well as the cotton sample found in India dating 300 BC [9]. Rhubarb belongs to rheum family (*Rheum palmatum* L., *Rheum rhabarbarum* L., *Rheum rhaponticum*) and contains mainly emodin. Rhubarb has been used for textile dyeing and provides yellow to orange colour with alum mordant, and a red hue with chromium mordant [9]. The roots of flowering plant; Indian mulberry known as al or suranji (*Morinda tinctoria* L., and *Morinda citrifolia*) contain mostly morindone which is used for dying textiles in India [10].

The dye components in natural dyes from animal origin also have mostly anthraquinone chromophoric groups. For example, the main colourants extracted from the following insect's kermes (*Kermes vermilio* Planchon and *Coccus ilicis*), cochineal (*Dactylopius coccus* Costa) and lac bug (*Laccifer Lacca* Kerr) are kermesic acid, carminic acid and laccaic

acid, respectively. These anthraquinone dyes with different mordants provide different colours. For example, most of them give the sharp red colour with a tin mordant; aluminum mordant gives a different range of red hue from pink to blue violet [6]. Kermes or crimson dye is obtained from female insects belonging to kermes family. These insects live on the oak tree in the Mediterranean. In order to produce 1kg of kermes dye, 150,000 insects should be boiled and after drying the residue should be ground. Kermes comes from the Arabic word “kerm” for worm which is vermiculus in Latin. Carmine and carmison are obtained from kermes insects. The red dye in kermes is kermesic acid. Kermes was used in ancient times in India to dye silk as well as in Greek and Rome. It was also used in middle ages as luxury dye known as “Cardinal’s Purple” [9].

Fabrics dyed with kermes dye have brighter colour compared with the madder dyed fabrics, also their wash fastness is better than samples dyed with cochineal dye [11]. Cochineal was used by Aztecs in Mexico and introduced to Europe by Spanish people [9]. The same as kermes, cochineal is extracted from the insects that live on cacti, and its main component is carminic acid. It should be noted that Polish cochineal (*Porphyrophora polonica*) contains both carminic and kermesic acid [4]. This is the dye used to provide the bright red colour of the tunic of the guardian soldiers in London in the 20th century [9].

The anthraquinone dyes extracted from plants and animals with their substituents are listed in **Table 1.2**. From this table it can be concluded that the plant origin red dyes have the substituents in one aromatic ring (R1-R4), except emodin, moridone and animal origin red dyes have substituents in both aromatic rings (R1-R4 and R5-R8).

**Figure 1.1** The basic structure of anthraquinone dyes**Table 1.2** The substituents of anthraquinone dyes

Colourant		Substituents							
		R1	R2	R3	R4	R5	R6	R7	R8
Plant origin	alizarin	OH	OH	H	H	H	H	H	H
	purpurin	OH	OH	H	OH	H	H	H	H
	pseudopurpurin	OH	OH	COOH	OH	H	H	H	H
	munjistin	OH	COOH	OH	H	H	H	H	H
	emodin	OH	H	OH	H	H	CH ₃	H	OH
	morindone	OH	OH	H	H	OH	CH ₃	H	H
Animal origin	kermesic acid	CH ₃	COOH	OH	H	OH	OH	H	OH
	carminic acid	OH	Glucose	OH	OH	H	OH	COOH	CH ₃
	laccaic acid	CH ₃	COOH	OH	H	H	OH	H	OH

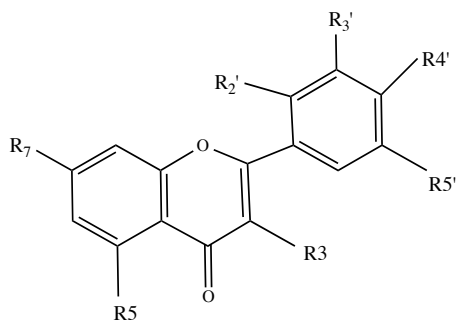
Flavonoid dyes

Flavonoid dyes are extracted from plants, fruits and trees. Flavonoid dyes are divided into 2 main classes of dyes: flavones and flavonols (3-hydroxyflavone) (see **Figure 1.2**) [12, 13]. The flavones are more resistant to photo degradation than flavonols [14]. The skeleton of flavones is 2-phenylchromen-4-one and the best-known dyes belonging to this group are apigenin and luteolin in both glycone and aglycone forms. The skeleton of flavonols is 3-hydroxy-2-phenylchromen-4-one and the best-known dyes belonging to this group are

rhamnetin, fisetin, morin, quercetin, kaempferol, myricetin. Flavonoids are used as mordant dyes and they can give several shades of yellow such as green-yellow to brown-yellow.

Weld also known as “dyer’s rocket, dyer’s weed, and dyer’s weld” is a dye extracted mainly from the leaves but also seed and flowers of the plant (*Reseda luteola L.*). Rocket comes from the French word roquette which refers to a herbaceous plant which are the plants without a wooden stem [15]. Weld contains mostly luteolin and some apigenin that belong to the flavones. The dye from fresh flowers and leaves provides a very bright fluorescent yellow colour. In Roman times the robes of the vestal virgins were dyed using weld. In the 13th century Lincoln green was also achieved by dyeing the fabrics with weld first and re-dyeing with woad. The dyeing process was improved by mordanting the fabrics using alum in the alkaline condition. The light-fastness of this dye is moderate and it shows a good wash-fastness in cold water. The berries from Persian berries and blackthorn (*Rhamnus*) are examples of flavonoids from fruit, and they are rich in rhamnetin [6], quercetin and kaempferol [12].

The following are examples of the flavonoids from wood. Fustic is a common name used for plants and the dyes extracted from these plants. The dye extracted from the wood of young fustic (also known as smoke tree) (*Rhus cotinus L.*) is mostly fisetin, while the main colourant in old fustic (known as dye’s mulberry) is morin. Also, the dye produced from the bark of quercetin (known as black oak) (*Quercus velutina*) is quercetin. Persian walnut (*Juglans regia L.*) contains myricetin [16]. **Table 1.3.** shows the chemical substituents of flavonoids.

**Figure 1.2** The basic structure of flavonoids**Table 1.3** The substituents of flavonoid dyes

	Colourant	Substituents						
		R3	R5	R7	R2'	R3'	R4'	R5'
Flavones	apigenin	H	OH	OH	H	H	OH	H
	luteolin	H	OH	OH	H	OH	OH	H
Flavonols	kaempferol	OH	OH	OH	H	H	OH	H
	quercetin	OH	OH	OH	H	OH	OH	H
	morin	OH	OH	OH	OH	H	OH	H
	myricetin	OH	OH	OH	H	OH	OH	OH

Homoisoflavonoid dyes

The dyes from brazilwood and logwood belong to homoisoflavonoids which has a 16-carbon skeleton with 2 phenyl rings and heterocyclic ring [5].

Brazilin dye is extracted from different species of Brazilwood known as Redwood or Sappanwood (*Caesalpinia crista*, *C. braziliensis*, *C. echinata*). The fabrics dyed with brazilin with aluminum mordant have red to violet colour [6]. It is worthy to note that brazilin oxidizes easily to brazilein and they are present in the dyed fabrics as a redox couple (the presence of oxidised and reduced forms of a compound together). Figure 1.3 (a) and (b) depict brazilin and brazilein, respectively. This dye was imported into Europe from Sri Lanka and East India. The Asian source was replaced with the Brazilian one when Portuguese explored South America and found the same tree. The quality of dye extracted from

Brazilian source is better than the Asian one. Brazilian woods are under protection due to exceeding extraction of dye [9]. The dyestuff extracted from the heartwood of logwood is haematoxylin. The chemical structure of haematoxylin is similar to brazilin except it has one more hydroxyl group at position 8 [12]. The application of different mordants in dyeing textiles with logwood different colours such as blue, violet and blue-black could be achieved [6]. Haematoxylin has different colours at different pH values. It is grey to purple in basic and red in acidic conditions, respectively. Logwood was introduced into Europe from Central America in wood pieces.

It is worthy to note that haematoxylin oxidizes easily to hematein and they are present in the dyed fabrics as a redox couple. **Figure 1.3** shows the chemical structure of these compounds.

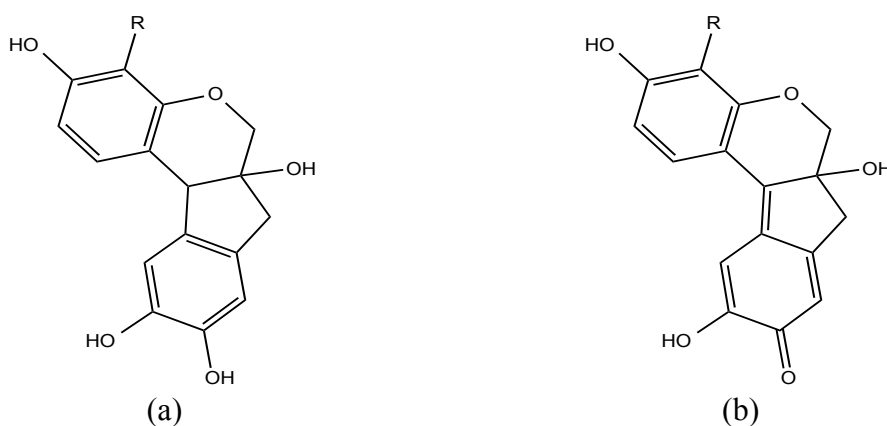


Figure 1.3 (a): The chemical structure of brazilin (R=H), haematoxylin (R=OH),
(b): brazilin (R=H), hematein (R=OH)

Quinoidic (quinoid) dyes

The skeleton of quinoidic dyes is the naphthoquinone. The representative plants for quinoidic dyes are alkanet, henna and green walnut shell. They are the source of alkannin, lawsone and juglone, representatively. **Figure 1.4** shows the chemical structures of naphthoquinone as well as the aforementioned quinoidic dyes.

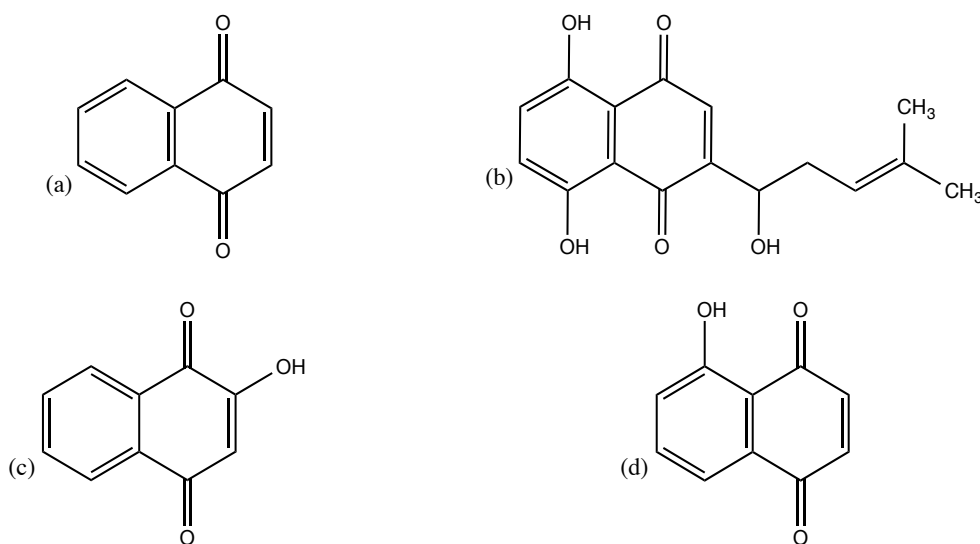


Figure 1.4 The chemical structure of quinoidic dyes: **(a):** 1,4-naphthoquinone, **(b):** alkannin, **(c):** lawsone and **(d):** juglone

Alkanet or dryer's bugloss (*Anchusa tinctoria* Lam.) has blue flowers and alkannin red dye is extracted from the roots. This dye shows low solubility in water and higher solubility in oil and alcohol. It provides purple colour in basic solution. It is used in mordant format to dye wool and silk. The mordants used for this purpose are alum and iron oxide. This dye has poor lightfastness [9]. Henna (*Lawsonia alba* Lam. and *Lawsonia intermis* L.) has lawsone dye. In ancient Egypt the leaves of henna were ground and used to decorate the bodies and dye the fingers and skin [9]. Also, it could be used to dye silk and wool with and without mordant to achieve orange to brown colours [17, 18]. Another source of quinoidic

dyes is black walnut (*julgans nigra L.*) containing juglone dye. This dye can be extracted from the leaves or from the dried shell of green walnut [19]. The following dyes could be detected in walnut bark extraction: quercetin, kaempferol, and dihydrokaempferol [8], Wool and silk dyed with walnut have brownish colour [20].

Carotenoid dyes

The best examples for carotenoid dyes are saffron (*Crocus sativus L.*) and annatto (*bixa orellana L.*) (**Figure 1.5**). The yellow colourant extracted from stigmas of saffron and the seeds of annatto known as crocetin and bixin, respectively. Since saffron is unstable it is rarely detected in archaeological artifacts [21]. Annatto gives orange colour to the fabric and in its mordant form the colour of fabric can be light orange to red orange [6].

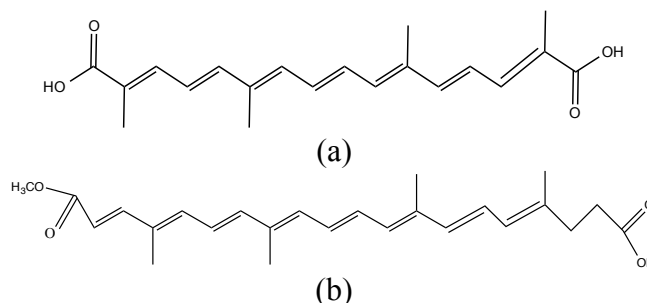


Figure 1.5 The chemical structure of (a): crocetin, (b): bixin

Chalcone dyes

The yellow colourant in florets of safflower (*Carthamus tinctorius L.*) is carthamin (**Figure 1.6**), which oxidizes to form the red- orange carthamon. Safflower was one of the important sources of yellow dyes in ancient Egypt. To dye the fabrics by safflower, the florets were boiled, dried, then ground. The fabrics dyed in yellow colour do not have a good wash-fastness to compared the fabrics dyed in red (carthamon) [4] [2].

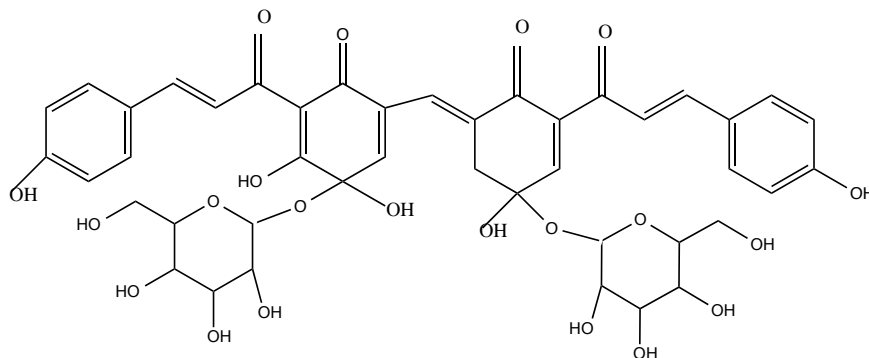


Figure 1.6 The chemical structure of carthamin

Indigoid dyes

The best well known plants for indigoid dyes are indigo (*Indigofera tinctoria* L., also known as *I. sumatrana*) and woad (*Isatis tinctoria* L.) [4]. The leaves of indigo plant and the bluish green leaves of woad contain indigo or indigotin (See **Figure 1.7**). As mentioned before indigoid dyes are examples of vat dyes.

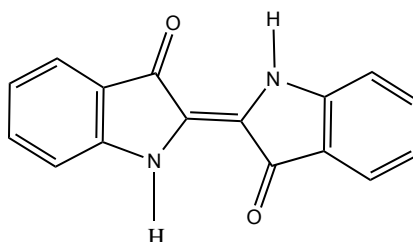


Figure 1.7 The chemical structure of indigo

Also, murex (6,6'-dibromoindigo) with animal origin belongs to indigoid dyes (See **Figure 1.8**).

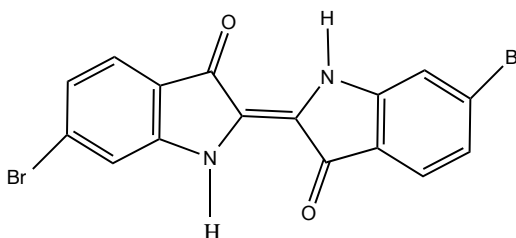


Figure 1.8 The chemical structure of murex or Tyrian purple

Aristotle in 4th century BC described the extraction method of murex from murex shell-fish. The other name of murex is Tyrian purple derived from the name of the Tyre city in Lebanon where ancient Phoenicians lived in the 20th century BC [9].

1.4 Identification of dyes

The identification of dyes is very important to different fields such as archaeology, conservation science and restoration. In regards to archeology, knowledge of the type of the colourant materials used for dyeing textiles or decorating objects helps to date the object. This aids in estimating how the knowledge of agriculture was developed at that time, or in tracing the trade road and find the dyes geographic origin. By identifying the dyes in the artifacts, conservation scientists would be better able to consider the best environmental conditions to preserve or exhibit the objects. Moreover, this information helps the restorer to use the same colourants applied in the original objects and choose the best restoration strategies.

In order to study the dyes in artifacts, the first approach is to choose a spot with a good colour distribution using either microscopic or colourimetric investigation [22]. Colourimetric studies can provide useful information on documentation of artifacts. This helps to monitor washing treatments and evaluate the effects of the environmental variations such as temperature and light on prolonged exhibition and storage of artifacts which can result in degradation or fading of colourants. Clementi *et al.* used this technique to study the time course of aging on alum mordanted and unmordanted wool samples dyed with madder. The results revealed that the mordant decreased the effect of degradation of dyes while oxygen had an accelerating role in degradation of colourants in madder [23].

To characterize the dyes on textiles, wet chemistry was used in the 1940s-1990s while in the 21st century instrumental analysis is the preferred method. In 1935 Pfister employed wet chemistry and published a systematic method to identify dyes in historical textile samples based on the changes in their colour and solubility at different pH values [6]. Schweppe also published several articles regarding wet chemistry in characterization of dyes in textiles [4], [24]. However, this method is not suitable due to the requirement of a large amount of sample (10 g textile) and lack of certainty in results [6]. Regarding the characterization of dyes using instrumental analysis, these analytical methods are usually spectroscopy and separation methods.

1.4.1 Spectroscopy

The basis for identification of dyes in spectroscopy is the comparison of spectra from the unknown with those of known samples.

The main spectroscopic techniques used in characterization of dyes and pigments includes UV-Vis spectroscopy, IR and Raman spectroscopy. UV-Vis spectroscopy can be divided into 2 groups: UV-Vis absorption spectroscopy and UV-Vis reflectance, which includes spectrofluorimetry.

The UV-Vis absorption spectroscopy (UVS)

When electromagnetic radiation shines on a sample, the molecules in the sample based on their structural groups absorb the light. UV-Vis light (200 to 800 nm) can be absorbed by complex molecules which have close electronic energy levels while those levels in simple molecules are widely separated and typically need higher energy than UV-Vis radiation for

excitation. Absorption of UV-Vis light causes electrons to elevate from ground state orbitals (σ , π and n) to the higher energy orbitals known as excited-state or antibonding orbitals (σ^* , π^*) [3]. The allowed transitions include: $\sigma \rightarrow \sigma^*$, $n \rightarrow \sigma^*$, $n \rightarrow \pi^*$, and $\pi \rightarrow \pi^*$. These transitions are measured by UV-Vis spectrophotometer and are known as absorption peaks. Absorbance is measured based on transmittance, by comparing the intensity of light before and after passing through a sample. The following formula shows absorbance:

$$A = \log \frac{I_0}{I} \quad \text{Eq. 1.1}$$

Furthermore, the UV-Vis spectrophotometer can be used to measure the reflectance of light from a sample surface. Intensity which is calculated as percentage (%R) is the ratio of intensity of reflected light from a sample (I) and the intensity of light reflected from a reference sample (I_0) [25].

Generally, it is considered as a destructive method when the dye is extracted from a large amount of sample using an organic solvent and taken up into another appropriate solvent (methanol) for UV-Vis absorption spectroscopy. Taylor studied red and blue colours on Scandinavian textile samples. The dyes for red and blue samples were thought to be madder and indigo [22]. Moreover, yellow and red Turkish textile samples from Topkapi palace were also investigated by this method. Concerning the red samples, some were dyed with madder, some with lac and the rest with cochineal. In case of the yellow samples their absorption were consistent with weld [26].

UV-Vis reflectance spectroscopy has been used since 1930 to study the %reflectance of pigments in different binding media [27]. This technique can be used with fiber optic probes, known as fiber optic reflectance spectroscopy; (FORS) to study *in situ* samples.

Leona *et al.* used FORS to study the blue and red pigments in Japanese paintings by Katsushika Hokusai. The blue colours were detected as indigo and Prussian blue. However, there was a doubt about the source of the red colour, it could be cochineal or lac dye [27]. FORS can be combined with other techniques to identify dyes and pigments. By means of UV-Vis reflectance spectroscopy, FT-IR and Raman, Maya blue pigment in the wall painting was identified as a complex of indigo and clay [28]. As mentioned above, colourimetry is considered as a reflectance method which can be used for colour mapping. Clementi *et al.* used this technique to study the time course of ageing on mordanted alum and unmordanted wool samples dyed with madder. The results revealed that the mordant decreased the degradation of dyes [29]. The recent work evaluating the weaknesses and strengths of using FORS on studying red, blue and yellow textile samples dyed with natural plants revealed that the red and blue colours could be detected successfully as alizarin, cochineal and indigo, while the yellow colours belonging the flavonoid group and curcumin could not be detected [30].

Fluorescence spectroscopy (FS)

Another spectroscopic technique is fluorescence spectroscopy (FS) also known as fluorometry, emission spectra are obtained by using a spectrofluorometer. In fluorometry, the UV-Vis light shines on a sample, which causes the transition of an electron to higher excited vibrational states (S_1 and S_2). The excited electron can lose energy and drop down to the lower states by 2 mechanisms: non-radiative and radiative transitions. Vibrational relaxation, internal conversion and intersystem crossing are examples of non-radiative transitions. If the electron drops from its highest to the lower excited vibrational levels it is known as vibrational relaxation which happens due to the dissipation of the energy of the

molecule to the surroundings (solvent) [25]. If the electron transfers from S_2 to any vibrational level in S_1 , it is known as internal conversion (IC). If the electron transfers from the lowest singlet excited state S_1 to a triplet excited state (T_1 : a state with a different spin multiplicity) it is known as intersystem crossing (ISC). The radiative transitions from vibrational levels in S_1 and T_1 to vibrational levels in S_0 are known as fluorescence and phosphorescence, respectively. In fluorescence studies the wavelength distribution and intensity of the emitted light is measured after excitation using a specific wavelength. Therefore, the emitted light always has lower energy than the excitation light. The intensity of the fluorescence light is calculated as shown in Eq.1.2:

$$F = \phi I_0 (1 - e^{-\varepsilon bc}) \quad \text{Eq.1.2}$$

ϕ = the quantum efficiency (the ratio of photons emitted per second to photons absorbed per second), I_0 = the intensity of incident light, ε = the molar absorptivity ($\text{l mol}^{-1} \text{cm}^{-1}$), b = the path length of the cell (cm), c = the concentration of the solution (mol l^{-1}) [31]. **Figure 1.9** explains the physics of fluorescence, which is known as the Jablonski diagram.

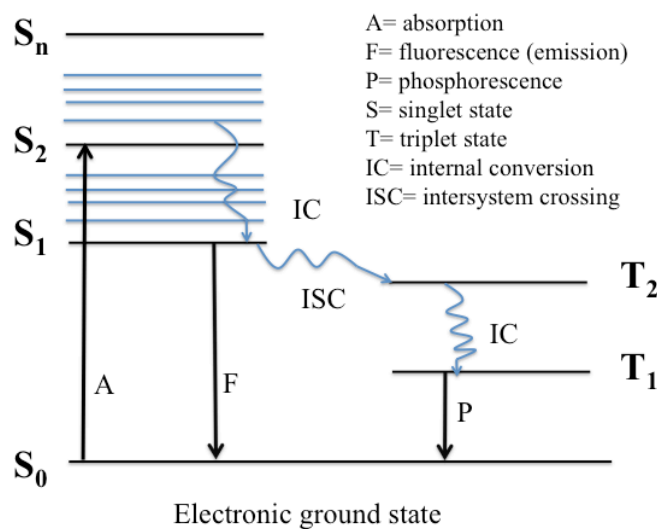


Figure 1.9 Jablonski diagram

This technique has been used to study the yellowing process of varnishes in paintings [32]. Also, the purple colour in the Greek marble basin was detected as madder by means of UVS, and FS and TLC, this study was a destructive technique since for all methods the sample was studied in solution form [33]. Fluorescence spectroscopy can be a non-destructive method when a fiber optic probe is used and it is known as fiber-optic fluorescence spectroscopy (FOFS) to collect the spectra. To study the colourants in madder and indigo, FS and UVS were used to collect the spectra from the samples in solution, and FOFS was used as an *in situ* technique to collect the spectra from the paint layers [34]. Claro *et al.* used UVS, FOFS to study the lake pigment based on alizarin and purpurin in different media: linseed oil and gum Arabic [35]. Clementi *et al.* used FOFS and HPLC-PDA to study the effect of mordant on the rate of aging of wool samples dyed with madder [23]. Moreover, this group used the same techniques to study the red and blue colours in 3 Renaissance tapestry samples, and could assign oreicin for red colours and indigo for blue ones [36]. They used UVS, FS and FOFS to study the wool samples dyed with oreicin and the red colour in the Renaissance tapestries and believe “In order to achieve a reliable identification of dyes on artistic objects a wide database of dyes on different substrates is necessary. Therefore, for investigating artistic textiles, standards of threads dyed using various dyes and different dyeing techniques need to be prepared” [37]. One of the main problems with FS is the lack of specificity of this technique for identifying dyes [17].

Infrared spectroscopy

Infrared (IR) spectroscopy can be used in 3 modes: absorption, reflection and transmittance. When the electromagnetic radiation in the IR range shines on the molecules with IR active bonds (their dipole moments should change during vibration) it absorbs IR radiation.

It means the amount of energy in each photon is sufficient to interact with the molecule and cause vibrational changes; however, it is not enough for electronic transition. The vibrations can be bending, stretching, or a combination of these vibrations. Therefore, the IR spectrum is achieved if the molecules have IR active bonds and its vibrational frequency matches with energy of IR radiation. Hook's law defines the vibration of covalent bond between 2 atoms (m_1 and m_2), which is shown in Eq.1.3.

$$\bar{\nu} = \frac{1}{2\pi c} \sqrt{\frac{k(m_1 + m_2)}{m_1 m_2}} \quad \text{Eq. 1.3}$$

where, $\bar{\nu}$ = vibrational frequency (cm^{-1}), c = the speed of light (cm s^{-1}), k = force constant (dyne cm^{-1}), m_1 and m_2 = the masses of 2 atoms bonded to each other (g) [38].

Different molecular structures vibrate at specific wavelengths or frequencies which help identify them. In Fourier transform infrared (FT-IR) an interferometer produces a signal containing all IR frequencies. By using this device all IR frequencies can be measured simultaneously in about 1 second. FT-IR has been used to identify dyes, binders and fibers in conservation studies of paintings and textiles [39], [40]. Low *et al.* used FT-IR to study the chemical structure of some dyes: alizarin; purpurin in their pure form as well as in madder lake form (a complex of organic dye with alum) and indigo. Also they investigated the changes in linseed oil after exposure to UV light in different intervals by FT-IR [41].

Shearer *et al.* employed FT-IR to define the date of creation of Virgin and Child, which is a panel painting belonging to a collection of the Clark Art Institute, attributed to 15th century. Since the blue dye was identified as Prussian blue, which was invented in 18th century and the binder was identified as oil or resin, which was different from egg tempera binding medium; the common binder used in most Italian 15th century paintings, there is

no doubt that this painting does not belong to 15th century [42]. Peruvian textile samples were investigated by IR and UVS. These studies revealed that the yarns were a mixture of cotton and animal source fiber, which attributed of the textiles to prehistoric people from Paracas. The blue and red dyes on these samples were identified as indigo and purpurin [43]. FT-IR was used to investigate a fiber and blue dye on archaeological samples belonging to Leicestershire. The fiber did not match the library references but the dye was assigned as indigo [44]. The main challenges with these vibrational techniques are that in many cases the substrate (fiber, painting media) has strong IR absorption [5]. Also in most textile samples the amount of dye is lower than the detection limit of FT-IR therefore the dyes cannot be detected [24].

Raman spectroscopy

Raman spectroscopy like IR is a vibrational spectroscopy. When electromagnetic radiation in the IR range shines on a molecule with Raman active bonds (their polarisability should change during this vibration), it vibrates the bond, which scatters the light. Therefore, as opposed to IR the molecule does not need to have a permanent dipole moment. IR and Raman spectroscopy are used as complimentary techniques, because the bonds showing strong IR absorption have a very weak Raman scattering and vice versa. Raman is a technique based on light-scattering and as opposed to IR technique there is no need for the energy of the incident light to be equal to the discrete energy level in the electronic state. The main difference between Raman and IR is in the nature of molecular transition. Raman transition can be seen in the molecules whose polarizabilities change during Raman transition, in other words the electron of the molecule should show the positional changes while

in IR transition the dipole moment of the molecule should be changed. In Raman the relative frequencies at which the molecule scatters the light are measured while in IR the absolute frequencies at which the molecule absorbs are measured. The incident light used in Raman is a monochromatic light which can be near UV, Vis, and near IR, while in IR spectroscopy the monochromatic light belongs to the IR region of magnetic spectrum [45].

Figure 1.10 shows IR transition, Raman transitions: they classified as rayleigh, stokes and anti-stokes, and fluorescence.

Raman spectroscopy has been used to identify dyes and pigments in paintings [46], [47], dyes on textiles [48], [49] and investigate fibers [50], [51] by recording the vibration of chemical bonds. Raman spectroscopy was employed to study the pigments and their biodegradation compounds found in the prehistoric cave paintings located in the USA [52]. The red pigment in 11th century Italian limestone corbel was identified as red ochre by using Raman spectroscopy [46].

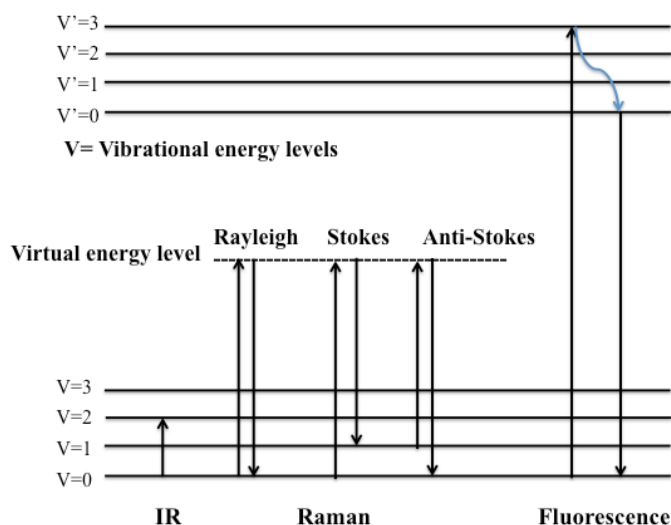


Figure 1.10 IR, Raman and fluorescence transitions

Moreover, the pigments in some prehistoric and Greco-Roman periods funerary Egyptian items including coffin, the covering mask and shroud were investigated. The red, blue and yellow pigments were identified as hematite and cinnabar, and Egyptian Blue and orpiment, respectively. The presence of cinnabar at that period of time can be considered as proof of the influence of Roman culture on Egyptian culture between 300 BC to 300 AD and there was a trade between these 2 countries [53].

Muralha *et al.* used Raman microscopy to identify 5 pigments on Persian manuscript miniatures belonging to the 16-17th century. The blue, yellow, red and black pigments were assigned as lazurite, orpiment, red lead and vermilion, and a carbon-based black pigment, respectively. The green pigments had the intense Raman spectra and could not be identified [54]. By using Raman and IR spectroscopy as non-destructive methods the blue dye in a plant as well as on silk and cotton textiles were identified as indigo [55].

The light source in Raman spectroscopy is a laser, which can cause fluorescence of the organic components in the sample. This makes it difficult or impossible to distinguish the dye from the fiber, especially if there is a complex matrix such as a mixture of dyes and their degradation products [17], [56], [57]. Moreover, surface-enhanced Raman spectroscopy (SERS) was used for studying dyes on textiles [58], and identifying the sepia dye on artifacts. Sepia dye is a red dye extracted from cuttlefish used as writing ink in the Greco-Roman civilization. It was detected using a diode laser at 785 nm and argon ion laser at 514 nm [59].

X ray fluorescence

X ray fluorescence (XRF) is a non-destructive elemental analysis technique [60]. This technique is based on the ejection of an electron from its atomic orbital by absorption of a light

wave (photon) of sufficient energy. The energy of the photon must be greater than the energy with which the electron is bound to the nucleus of the atom. When an inner orbital electron is ejected from an atom, an electron from a higher energy level orbital will be transferred to the lower energy level orbital. During this transition, a photon may be emitted from the atom. This fluorescent light is called the characteristic X-ray of the element. The energy of the emitted photon will be equal to the difference in energies between the 2 orbitals occupied by the electron making the transition. The energy difference between 2 specific orbitals in a given element is always the same the photon emitted when an electron moves between these 2 levels, will have the same energy. Therefore, by determining the energy (wavelength) of the X-ray, it is possible to determine the identity of that element. For a particular energy of fluorescent light emitted by an element, the number of photons per unit time (generally referred to as peak intensity or count rate) is related to the amount of that element in the sample. By determining the energy of the X-ray peaks in a sample's spectrum and by calculating the count rate of the various elemental peaks, it is possible to qualitatively establish the elemental composition of the sample and to quantitatively measure the concentrations of these elements. Due to instrumental limitations and low X-ray fluorescence yields, it is often difficult to quantify elements lighter than sodium ($Z = 11$) [40].

X ray fluorescence is a common technique for studying metal composition of coins [61], sculptures [62] and metal threads in textiles [63]. Pigments used in paintings [64], [65], inks [66], [67], and ceramics have also been studied by XRF. Black pigments in 75 ceramics from 5000-325 BC in the Nicolas Museum in Cyprus were studied using XRF technique. It was concluded that the black pigments in artifacts belonging to 5000 to 1625

are rich in iron pigments while those objects belonging to 1050 to 325 BC are rich in manganese [68].

1.4.2 Separation techniques

As mentioned before textiles are made of organic fibers that can interfere with dye when analyzing with IR and Raman techniques. Therefore, separation methods are employed to eliminate the fiber interferences. A number of separation techniques have been explored and these include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE).

Prior to analytical separation dyes need to be extracted from the substrate. The extraction efficiency is related to the solvent as well as time, temperature and mechanical agitation. Regarding the role of solvent in extracting dyes, the Hansen solubility parameter (HSP) can be used to explain why some solvents extract more dyes than the others. Hansen solubility index is based on 3 molecular interaction forces: Van der Waals interaction force, electrostatic interaction force, and hydrogen bonding. If 2 molecules have similar HSP values they are alike and the solvent can dissolve the solute. In Chapter 5 HSP will be explained in details.

In chromatography analytes are separated based on their concentration equilibria between 2 phases (stationary and mobile). The stationary phase is fixed and the analytes carried by the mobile phase through the medium can be liquid or gas. The affinity between stationary phase and analytes improves the separation by slowing down the movement of target analytes in the mobile phase.

Thin layer chromatography

In thin layer chromatography (TLC) the stationary phase can be a plate of glass or aluminum foil, which is covered by adsorbent materials such as silica gel and aluminum oxide. A mixture of samples are put on the spotting line on the plate by using capillary tube and by placing the plate in a closed eluting system containing a proper solvent (usually this is a mixture of polar and nonpolar solvents), the analytes are separated due to different affinity to stationary and mobile phases. The analytes then are characterized by comparison of the distance of the spot moved from spotting line to solvent front line and the colour of spots with those of reference samples [7].

Thin layer chromatography (TLC) has been employed for studying the dyes on textiles [69], [70], [71] and paper [72]. Thin layer chromatography (TLC) compared to other analytical methods is fast, cheap and simple. By applying TLC using polyamide plates Schweppe could identify carminic and kermesic acids in a piece of red satin from Italy belonging to 16th century. It indicated that the fabric was dyed with Polish cochineal. Moreover running TLC on the dyes extracted by dimethylformamide from a piece of Ushak carpet belonging to late 15th century showed that the dyes used for red and brownish-yellow threads were madder and weld, respectively. In these examples the amount of textile sample was not indicated [4]. Kharbade *et al.* used TLC to investigate the red threads from Indian textile samples dated 19th century. The chromatograms of some red textile samples were identical to that of wild madder (containing munjistin), and Indian mulberry (containing morinda) was assigned for other red samples [10]. A mixture of some dyes cannot be separated completely by TLC which is the weakness of this procedure [73].

High performance liquid chromatography

In high performance liquid chromatography (HPLC), the stationary phase is a column with 3 to 30 cm length and 1 to 5 mm diameter; made of stainless steel, glass, or polymers such as polyetheretherketone (peek) [74, 75]. The column is packed with either porous particles, made of silica with 3 to 10 μm diameter, or with particles made of glass and polymers with 30 to 40 μm diameter which are surrounded by resin. The mobile phase in HPLC is a liquid, which is mostly a homogenous mixture of water with polar organic solvent such as methanol or acetonitrile. The samples in HPLC can be liquid or if they are solid they can be dissolved in a proper solvent and injected into HPLC for separation. In HPLC solvent flows in the column by the aid of a pump system.

High performance liquid chromatography is a common technique to study pigments [76], binding media [77], [78], tannins [79], resins [80], and dyes from textiles [76] in the field of cultural heritage.

High performance liquid chromatography can be equipped with many different detection schemes including UV-Vis light, photo diode array (DAD), fluorescence detection, and mass spectrometry (MS) to study dyes from textiles or paintings. Regarding the application of UV-Vis light only specific compounds that can absorb light at specific wavelength can be detected while photo diode array detector due to having a large number of diodes can detect more than one absorbing eluent at different wavelengths simultaneously. HPLC equipped with UV-Vis detection scheme was used to study a 2000- year -old purple textile from Israel. It was revealed that the fabric was dyed with *Murex trunculus* mollusk [81]. HPLC with DAD was employed to study red textile fragments. Alizarin, purpurin and kermesic acid could be identified in 13th century in a bishop tunic, which indicated 2 sources of anthraquinone had been used: madder and kermes. The analysis of red dyes in

17th century Coptic textile showed that it had mostly purpurin and less amount of alizarin, however pseudo purpurin and munjustin were detected as co-eluted fractions [76]. Moreover, the blue and purple colourants of reference samples dyed with indigoids, and 1st century BC textile sample were separated and identified as indigotin, indirubin, 6- monobromoindigotin and 6,6'-dibromoindigotin by HPLC-DAD [82], [83].

A comparison study was done on textiles from pre-Columbian Peruvian by using HPLC-DAD. The red dyes from different pre-Columbian cultures were identified as cochineal (contains carminic acid), and relbunium (contains purpurin) where they used individually or as a mixture. In some samples the mixture of luteolin and xanthopurpurin were found but the plant could not be identified [84]. HPLC-MS, which is also known as LC-MS has been used to study the dyes on fabrics. Yamaoka *et al.* investigated alizarin as the main red colourant on Greco-Roman textiles [85]. LC-MS was employed to study flavonoid colourants of yellow dye extracted from the wool dyed with weld and onion skin [86]. Moreover, this research group used the same technique to study the photo-degraded compounds of morin and quercetin on the dyed alum mordanted wool, which was identified as dihydroxybenzoic acid [87]. Szostek *et al.* analyzed the dyes extracted from a Coptic textile using of LC-MS and LC-UV-Vis and compared the detection limits of these techniques. The detection limit for purpurin, alizarin, and indirubin was the same in both techniques, while the results obtained using LC-MS for luteolin, apigenin, and rhamnetin showed better detection limits [88]. Yellow silk samples were analyzed with LC-MS and flavonoid O-glycosides and curcumin were identified as the source of yellow dyes from pagoda tree and turmeric, respectively [89]. Furthermore, LC-MS coupled with HPLC-DAD were employed to study the dyed yarns of the Portuguese 17th century carpet. This study showed

that the yellow and blue dyes were weld and indigo, respectively. However, LC-MS was not able to detect the source of red dye, which was known as brazilwood. It seems this dye is not photo resistant and discoloured. This study was used to compare the dyeing protocols of yarns in 17th and 19th centuries [90].

Gas chromatography

In gas chromatography (GC) the stationary phase is a column made of stainless steel or glass coil construction with 1 to 100 m length, packed with adsorbent particles. The size of the particles are between 0.1 to 8 μm . In GC the mobile phase is gas and the samples should be injected into GC in this form. Therefore, GC is a more convenient technique for volatile samples, while liquid and solid samples should be heated at high temperature to evaporate volatile components from the samples [91].

Gas chromatography (GC) has also been used to study the organic materials such as balms from mummification, wax sculptures, adhesives, binders used in paintings [92]. GC-MS has been used since 1990 in the field of conservation by other researchers, particularly by Colombini *et al.* They used this method to study proteinaceous binder used in the historical fresco in Pisa. The ratio of amino acids as degradation products was used to conclude that casein was the main binder material [93]. Furthermore, in another study of wall painting in Arezzo (Italy) the same technique was used and they could confirm the application of the mixture of 2 different kinds of binders, which is known as “tempera grassa” the emulsion of egg yolk and linseed oil [94]. GC-MS was employed to identify the varnishes used in the 13th century Italian paintings as mastic and dammar. The presence of mastic contributed to the original work while the dammar was referred to the restoration

done in 20th century [95]. The pigments and the binders used in an ancient Greek polychromy were also investigated using different methods including GC-MS. The pigments were identified as madder lake and shellfish purple by HPLC equipped with UV-Vis detection scheme. The results for binder from FTIR, and GC-MS were in agreement and it was detected as egg tempera [96]. The identification of micro samples of organic paint binders is still under investigation [97]. As mentioned before, the dyes can be investigated by GC but the same as binder materials, they need to be derivatized as they are often large, polar molecules [98].

Capillary electrophoresis

Capillary electrophoresis (CE) is a technique whereby ionic species are separated under electric field, in a capillary tube (stationary phase) containing buffer (mobile phase) [25]. Anions migrate toward anode and cations migrate toward cathode. This technique can be used for separation of small ($M_r < 2000$) and large molecules ($2000 < M_r < 100\,000$) [99]. The capillary tube is fused silica capillary with 10-100 μm I.D. (inside diameter) and 30-100 cm length. In the next sections the following subjects are discussed: brief information on the principal and concepts of CE, separation modes of CE, and the application of CE in art.

1.5 Capillary Electrophoresis (CE): Principle and concepts

Jorgeson and Lukas introduced capillary electrophoresis (CE) in early 1980 and since that time it has been used widely for the separation of organic and inorganic compounds [100].

Phoresis in Greek means “being carried” and electrophoresis is a technique whereby ionic species are separated under electric field while CE is the electrophoresis performed in a capillary tube. **Figure 1.11** depicts the schematic of CE instrumentation.

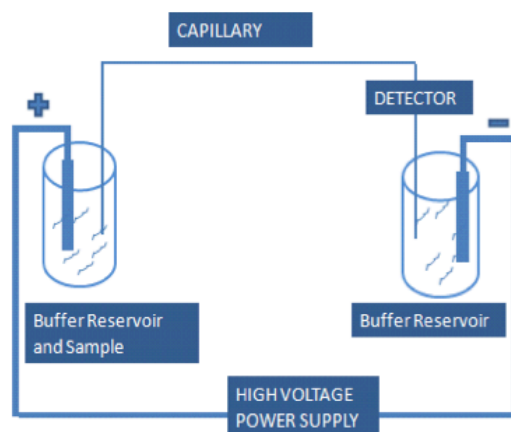


Figure 1.11 The CE instrumentation

In general the CE instrumentation consists of the following parts: a fused silica capillary tube (inside diameter: 10-100 μm , length: 30-100 cm), high voltage power supply (HVPS) which generates voltage: 0-30 kV, 2 electrodes: anode; the positive electrode, and cathode; the negative electrode, 2 buffer reservoirs, a detector which can be on-column detector which is placed at capillary known as end-column detector [101]. The detectors are mostly used for absorption [102] and fluorescence detectors [103].

The principle of CE is based on the migration of anions toward the anode and migration of cations toward the cathode in a buffer solution in a capillary tube. The buffer solution is known as background electrolyte (BGE) or running buffer. The materials, which have been used to manufacture capillary tubes, are Teflon and borosilicate glass [104]. Teflon and borosilicate glass are replaced with fused silica due to its unique properties such as transparent to a wide range of wavelengths, high thermal conductivity, and capability to fabricate the tubes as narrow as a few micrometers. The inner surface of fused silica capillary

in alkaline condition forms a layer of negative silanol groups [105], and by absorption of buffer cations to this layer a diffuse double layer or electrical double layer forms. The outer layer is mobile and solvated. Therefore by applying an electric field this layer is pulled towards the cathode, which causes the electroosmotic flow (EOF) of buffer (See **Figure 1.12**).

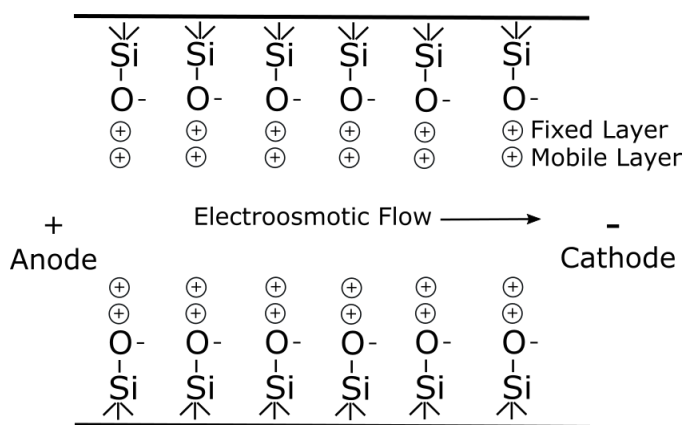


Figure 1.12 The structure of fixed and mobile layers formed inside the capillary tube.

The sample can be injected into capillary either by electrokinetic or hydrodynamic injection. In electrokinetic injection samples are introduced into the capillary from the anode side, by applying a voltage. The amount of sample introduced into the capillary via electrokinetic injection depends on the following factors: applied voltage, time of injection, mobility of eluents and diameter and length of capillary. Therefore, this injection method is selective meaning the more mobile eluent is injected than the less mobile one. Also, using applied voltage to introduce the sample into capillary can change the chemical structure of eluents; therefore, for each run a fresh solution should be prepared. In hydrodynamic injection, samples are introduced to the capillary by applying vacuum or pressure at one end of capillary and the amount of sample injected to the capillary is related to: the applied

pressure, injection time, the viscosity of BGE and diameter and length of capillary. One of the advantages of using the hydrodynamic injection method over the electrokinetic one is that the amount of sample injected via hydrodynamic injection is more precise. Also, the pressure does not change the chemical structure of compounds; therefore, the same sample vial can be used for several analyses. Introduction of very viscous eluents using hydrodynamic injection is very problematic. In spite of the drawbacks of electrokinetic injection over hydrodynamic, electrokinetic injection is mostly used due to its ability for sensitivity enhancement. This can be explained by the amount of the analytes introduced into the capillary. In hydrodynamic injection, the volume injected contains analytes and BGE, but in electrokinetic injection the volume injected contains more analytes than BGE while the introduction is based on the electrophoretic and electroosmotic mobility. It is reported that the sensitivity could be improved 100 to 1000 times when electrokinetic injection was used [106].

1.6 The Basis for Electrophoretic Separation

The separation is based on the apparent mobility of the analytes, which is the vector sum of the electrophoretic mobility and electroosmotic mobility [107].

1.6.1 Electrophoretic mobility μ_{ep}

Generally speaking, mobility is a characteristic of analyte, which is the ratio of the velocity of the analyte *Eq. 1.4*, to the electric field strength *Eq. 1.5*.

$$v_{ep} = \frac{L_d}{t_m} \quad \text{Eq. 1.4}$$

Where: v_{ep} = electrophoretic velocity (cm s^{-1}); L_d = length of the capillary to the detector (cm), in this study $L_d = L_t$; while L_t = total length of the capillary (cm); t_m = time of migration (s)

$$E = \frac{V}{L_t} \quad \text{Eq. 1.5}$$

where: E = electric field strength (Vcm^{-1}); V = applied voltage (V); L_t = total length of the capillary (cm)

In the case of the electrophoretic mobility, as shown in Eq. 1.6, it is defined as:

$$\mu_{ep} = \frac{v_{ep}}{E} = \frac{\frac{L_d}{t_m}}{\frac{V}{L_t}} = \frac{L_t^2}{t_m \times V} \quad \text{Eq. 1.6}$$

Where: μ_{ep} = electrophoretic mobility ($\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$); v_{ep} = electrophoretic velocity (cm s^{-1}); E = electric field strength (Vcm^{-1})

Also, electrophoretic mobility can be described by using the definition of constant velocity of an analyte in an electric field. When the analyte moves with a constant velocity it means the electrostatic attraction force and retarding frictional force, which drag the analyte toward opposite directions are in equilibrium. Electrostatic attraction force is conveyed by the electric field and depends on the charge on the analyte and electric field strength [108] Eq. 1.7:

$$F = qE \quad \text{Eq. 1.7}$$

Where: F = the force of electrostatic attraction (N); q = the charge on the ions in the sample (C); E = electric field (Vcm^{-1})

Conversely, retarding frictional force depends on the electrophoretic velocity v_{ep} and friction factor; f as shown in Eq. 1.8

$$F_f = v_{ep} f \quad Eq. 1.8$$

where: F_f = retarding frictional force (N); v_{ep} = electrophoretic velocity (cm s^{-1}); f = friction factor.

The friction coefficient related to the hydrodynamic radius, r of the ion and the viscosity, η , of the surrounding medium *Eq. 1.9*.

$$f = 6\pi r \eta \quad Eq. 1.9$$

Where r = ionic radius of the ions (cm); η = viscosity of the solution (cP)

Therefore, the retarding frictional force can be defined by introduction of *Eq. 1.8* in *Eq. 1.9* which leads to *Eq. 1.10*.

$$F_f = v_{ep} 6\pi r \eta \quad Eq. 1.10$$

At a given velocity the force which is conveyed by the electric field is equal to the retarding frictional force, therefore, $F = F_f$ and by substitution of F by *Eq. 1.7* and F_f by *Eq. 1.9* we will have *Eq. 1.11*:

$$qE = v_{ep} 6\pi r \eta \quad Eq. 1.11$$

From *Eq. 1.11*, q is defined as $q = \frac{v_{ep} 6\pi r \eta}{E}$, and from *Eq. 1.6* we know that v_{ep}/E is μ_{ep} therefore, $q = \mu_{ep} 6\pi r \eta$, and we will have *Eq. 1.12* as follows:

$$\mu_{ep} = \frac{q}{6\pi r \eta} \quad Eq. 1.12$$

Where, μ_{ep} = electrophoretic mobility ($\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$); r = ionic radius of the ions (cm); η = viscosity of the solution (cP)

According to *Eq. 1.12* the electrophoretic mobility is proportional to the ion charge q and inversely proportional to ion size r and the viscosity of the solution. Viscosity is temperature dependent, decreasing with increasing temperatures. Therefore, by increasing the temperature, electrophoretic mobility will increase [108].

1.6.2 Electroosmotic mobility μ_{eo}

The movement of liquids relative to a stationary charged surface, such as the membrane, capillary tube, and porous material, under the influence of the electric field is known as electroosmotic velocity (v_{eo}) or electroosmotic flow (EOF) or endosmotic migration. Since mobility is the velocity over the electric field, the electroosmotic mobility is defined as [108]

$$\mu_{eo} = \frac{v_{eo}}{E} \quad \text{Eq. 1.13}$$

Where: μ_{eo} = electroosmotic mobility ($\text{cm}^2 \text{V}^{-1} \text{s}^{-1}$); v_{eo} = electroosmotic velocity (cm s^{-1}); E = electric field strength (V cm^{-1})

Also, electroosmotic mobility can be defined by [107]

$$\mu_{eo} = \frac{(\epsilon \zeta)}{4\pi\eta} \quad \text{Eq. 1.14}$$

Where: ϵ = medium dielectric constant; ζ = zeta potential at the capillary–buffer interface (mV); η = viscosity of the medium (cP)

As mentioned before, the apparent mobility is the vector sum of electrophoretic mobility *Eq. 1.12* and electroosmotic mobility *Eq. 1.14*, and from these equations it can be concluded that the average rate of migration is related to the average charge on the ion, the average size of the ion, and the average of voltage applied. From this information it can be

concluded that electroosmotic flow (EOF) can be affected by the following factors: the pH, concentration, viscosity of the buffer, temperature, field strength, the presence of surfactants and modifiers, as well as the charge on the capillary wall [109].

1.7 Joule heating (H)

The migration of ions, which is known as ionic conduction can occur via 3 ways: the analytes can move under the influence of applied voltage through the bulk electrolyte due to their electrophoretic mobility. They can be carried by convection of bulk electrolyte, which is driven by applying voltage. Also they can be transferred by electroosmotic flow of the electrolyte, which is generated due to the formation of double layers on the capillary walls [110]. The conductivity of an electrolyte in a filled capillary tube is expressed in *Eq. 1.15*.

$$\sigma = \frac{iL}{VA} \quad \text{Eq. 1.15}$$

Where, σ = conductivity (Sm^{-1}), i = current (A), L = length of capillary (m), V = applied voltage (V), A = cross sectional of capillary (m^2).

In general, the resistance of an electrolytic solution to the flow of current generates heat.

The heat is known as “Joule heating” [107] and it can be calculated by using *Eq. 1.16*.

$$J(t) = iVt \quad \text{Eq. 1.16}$$

where, $J(t)$ = generated heat (Joules) as a function of time (seconds). Introduction of *Eq. 1.15* in *Eq. 1.16* leads to *Eq. 1.17*, which shows how Joule heating is related to the ionic conduction, length and cross section of capillary [111].

$$J(t) = \frac{\sigma AV^2 t}{L} \quad \text{Eq. 1.17}$$

Joule heating causes thermal gradients across the capillary, which can result in bubble

formation due to vaporization of solution. Therefore, by decreasing the applied voltage and increasing the length of capillary, as well as using a low conductivity buffer solution the amount of Joule heating will decrease effectively. The narrow bore capillary with a 10-100 μm inside diameter provides a large surface area-to-volume ratio, which helps to dissipate Joule heating more efficiently [112]. Therefore, the narrower capillary allows a higher electric field to be applied, which leads to a fast separation with better resolution.

1.8 Separation efficiency

The efficiency of separation techniques is described as the “number of theoretical plates” (N), which are the discrete sections where separate equilibrations of the analyte between the mobile and stationary phase occur. There are several factors of dispersion of analyte, which cause peak broadening or peak dispersion (σ^2) such as: Joule heating, capillary wall binding, and diffusion. During the migration of analyte, if diffusion (D) is considered as the only factor of the peak broadening, it can be calculated as shown in *Eq. 1.18*, which was described by Einstein [107].

$$\sigma^2 = 2Dt_m = \frac{2DL^2}{\mu_{ep} V} \quad \text{Eq. 1.18}$$

Where D = diffusion ($\text{cm}^2 \text{s}^{-1}$), t_m = migration time (s) and the other terms are as before

The number of theoretical plates is given by *Eq. 1.19*:

$$N = \frac{L_t^2}{\sigma^2} \quad \text{Eq. 1.19}$$

By replacing *Eq. 1.6* and *Eq. 1.18* in *Eq. 1.19* the equation for the calculation of number of plates yields *Eq. 1.20*. It shows that the efficiency depends on electrophoretic mobility

of the analyte, applied voltage and diffusion.

$$N = \frac{\mu_{ep} V}{2D} \quad Eq. 1.20$$

1.9 Resolution

The resolution R_S , between 2 analytes is defined as *Eq. 1.21*:

$$R_S = \frac{1}{4} \frac{\Delta\mu_{ep} \sqrt{N}}{\mu_{ep} + \mu_{eo}} \quad Eq. 1.21$$

Where $\Delta\mu_{ep}$ = the difference between electrophoretic mobility of 2 analytes, μ_{ep} = the average of the electrophoretic mobility of 2 analytes, N = the number of theoretical plates.

By replacing *Eq. 1.20* in *Eq. 1.21*, resolution R_S can be calculated as *Eq. 1.22* [107].

$$R_S = 0.177 \Delta\mu_{ep} \sqrt{\frac{V}{(\mu_{ep} + \mu_{eo}) D}} \quad E q. 1.22$$

From this equation, it can be deduced that resolution is proportionally related to difference between electrophoretic mobility of 2 analytes and the square root of voltage. Therefore, in order to improve the resolution, increasing the difference between electrophoretic mobility of 2 analytes is more effective than increasing the voltage, since increasing the voltage can cause the Joule heating. Also, the faster migration of some analytes, which results in co-elution, can happen at higher voltages. Different separation modes of CE are based on controlling the mobility of analytes by using a buffer.

1.10 Separation modes

CE can be used in different separation modes, known as: capillary zone electrophoresis

(CZE), micellar electrokinetic chromatography, (MEKC) also known as micellar electrokinetic capillary chromatography (MECC), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE), and capillary isotachopheresis (CITP). Since, CZE and MEKC were the techniques used in the study of historical dyes in this project, discussion of these 2 separation methods will now follow.

1.10.1 Capillary zone electrophoresis (CZE)

The simplest form of separation in CE is known as capillary zone electrophoresis (CZE) or free solution CE. The analytes are injected into a capillary filled with running buffer and by applying high voltage, the electroosmotic flow (EOF) is generated from the anode towards the cathode. Separation is based on the charge- to- size ratio of analytes, and in other words, based on the differences between the electrophoretic mobilities of the analytes. The EOF can be affected by pH, therefore the separation should be done in a buffer solution. The most common buffers used in CZE are phosphate (pH= 1.14 - 3.14 and pH= 6.20 - 8.20), acetate (pH= 3.76 – 5.76), borate (pH= 8.14 – 10.14), and N-cyclohexyl-3-aminopropanesulfonic acid (CAPS, pH= 9.70-11.10) [113]. The elution order of the ionic analytes in CZE, which is related to, the mobility is as follows: cations first, then neutral species and anions are last (see **Figure 1.13**).

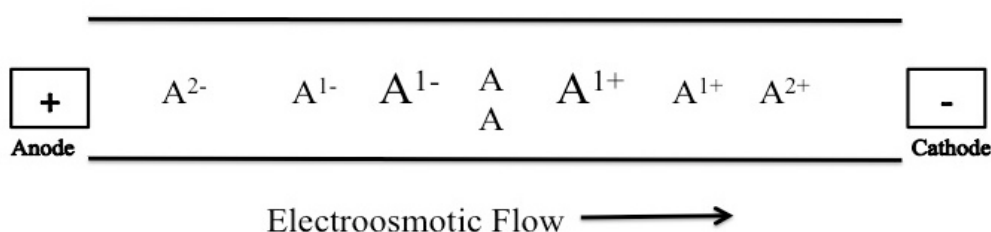


Figure 1.13 Order separation in CZE.

1.10.2 Micellar electrokinetic chromatography (MEKC)

Teraby *et al.* in the mid-1980s introduced micellar electrokinetic chromatography (MEKC) [114]. MEKC involves the addition of surfactants to running buffer to form micelles. When the concentration of surfactant is above the critical micelle concentration (CMC), the number of molecules known as the aggregation number (N_g) join together to form micelles. Micelles are the aggregation of long chain molecules (10 – 50 carbon chain) with a hydrophobic core and hydrophilic outer surface. There are 4 kinds of surfactants used in CE: anionic, cationic, zwitterionic and nonionic. Examples for these surfactants are shown in **Table 1.4** [115].

Table 1.4 The examples of different kinds of surfactants with their critical micelle concentration (CMC) aggregation number (N_g)

Surfactants	Example	CMC (mM)	N_g
anionic	sodium dodecyl sulfate (SDS)	8.1	62
	sodium tetradecyl sulfate (STS)	2.1	138
cationic	cetyltrimethylammonium bromide (CTAB)	0.92	61
nonionic	Polyoxyethyleneglycol dodecyl ether (Brij-35)	0.1	40
zwitterionic	sulfobetaine	3.3	55

The ionic and neutral analytes with molecular mass less than 2000 ($M_r < 2000$) can be separated using MEKC due to the partitioning mechanism of analytes between the micelles and running buffer. In the MEKC mode, the separation is achieved in a capillary tube under alkaline conditions by adding sodium dodecyl sulfate (SDS), which is the most widely used surfactant in MEKC [108].

At neutral to alkaline condition the electroosmotic flow is from anode to cathode, while the electrophoretic mobility of the SDS micelles is toward the anode. The elution order of analytes in MEKC is as follows: anions, neutral and cations. Anions elute first due to the

electroosmotic flow and the repulsion from the negatively charged SDS micelles. The separation of neutral analytes is based on their hydrophobicity and their migration rate is the same as EOF. The ion-pair attraction between cations and micelles causes the cations to elute last. (see **Figure 1.14**).

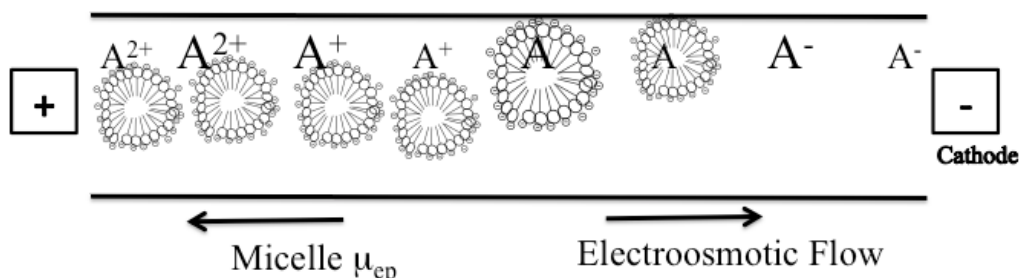


Figure 1.14 The migration direction of SDS and ionic analytes in MEKC

1.11 Application of CE in art

Some CE methods have been developed to characterize different types of binding media, such as plant gums, drying oils, animal glues, as well as natural resins, which have been used as adhesives, coatings and varnishes. Moreover, CE has been employed to investigate dyes and pigments, synthetic dyes as well as degradation products of textiles and paper and binding media. As mentioned before, GC is a popular technique to investigate binding media which needs derivatization, while the application of CE has an advantage over GC, which does not need the derivatization. This is due to the presence of functional groups, which can be ionized in the alkaline condition and migrate in a capillary. The plant gums are primarily made of polysaccharides with hydroxyl and acidic functional groups that can be ionized at pH over 12. Gröbl *et al.* employed CE equipped with UV-Vis detector to separate and identify the monosaccharides such as glucuronic acid and rhamnose in gum

arabic and galacturonic acid and fucose in gum tragacanth. The separation was done using a 36 cm capillary tube at 20 kV, in background electrolyte made of a buffer of 20 mM 2,6-pyridinedicarboxylic acid (PDC) at pH= 12 and 0.5 mM cetyltrimethylammonium bromide (CTAB) as a cationic surfactant to reverse the electroosmotic flow [116]. Drying oils consist of the esters of polyunsaturated fatty acids such as alpha-linoleic acid, linolenic, oleic, the degradation products of these compounds are mostly di-carboxylic acids. Since these compounds can form the anion analytes at intermediate pH values, they can be separated by CE. The constituents and degraded compound of the drying oil used in 2 paintings of 19th century could be detected using a 48.5 cm capillary. The separation was carried out in a background electrolyte (BGE) consisting of histidine buffer (pH = 5.85, 20 mM) containing 10 mM salicylic acid, at 10 kV and the analytes were detected both with CE equipped with UV and CE with conductivity detection. The binding media in both paintings were detected as palmitic, stearic acid linolenic acids. Moreover, the degraded compound in both samples was identified as azelaic acid [117]. The 3 main animal glues: egg, collagen and casein were studied by capillary zone electrophoresis (CZE). The separation of amino acid contents were investigated using a 60.2 cm capillary tube, at 20 kV, in a background electrolyte made of 51.9 mM chloroacetic acid and 3.05 mM lithium hydroxide at pH= 2.26, the analytes were detected by DAD [118]. This research group used the same conditions to study the ability of CE to separate a mixture made of different binders: plant gums, drying oil and animal glue [119]. The results were used to study the binder in an 18th century polychrome painted sculpture, and it was revealed that collagen was used as the main binder and could be separated from plant gum and drying oil binders by choosing an appropriate pH [120]. Moreover, the potential of CE to separate the natural resin such as

diterpenoic resins, balsams and copals in a mixture of binder media was investigated. The separation was carried out using a 40 cm capillary tube, at 20 kV in a background electrolyte made of a 20 mM borate buffer at pH= 9.25, the separation was done in 5 minutes and analytes were detected by diode array detector. The results were used to identify the resin used in a 19th century gilded frame as colophony or rosin [121]. Separation conditions of CE in different modes for identification of natural dyes separately also from paintings and textiles have been investigated [122-132].

The small red samples from a 17th century oil painting belong to Diocesan Museum of Holy Art of Bilbao in Netherlands were analysed using micellar electrokinetic chromatography (MECK) equipped with DAD. The optimum separation conditions were defined by working on reference samples prepared according to recipes from the 16th century. The separation was achieved in less than 13 minutes using a 58 cm capillary tube at 20 kV, in background electrolyte made of 20 mM borate buffer (pH= 9) containing 20 mM sodium dodecyl sulfate(SDS) and 10% acetonitrile as modifier. Three small separated peaks in the electropherogram of the red sample were assigned as alizarin, quinizarin and purpurin. It indicated the sample was dyed with madder [132].

Capillary electrophoresis has been employed to study the synthetic dyes used in textiles [133], [134], [135], [136], pen inks [137], [138] and colourful photographs [139]. Siren *et al.* used CZE equipped with UV to study the black reactive and black acid dyes extracted from cotton and wool fibers. The separation was achieved in 0.14 M 3-(cyclohexylamino)-l-propanesulphonic acid (CAPS) buffer at pH = 10.8 [134]. MEKC with UV detection was employed by Zlotnick *et al.* to investigate the optimum condition for separation of black

pen. The different concentrations of borate buffer at pH= 9.5, separately and in combination with different concentration of micelles, sodium dodecyl sulphate (SDS) were investigated. The effect of methanol as modifier at different concentrations (0%, 5%, 10% and 25%) and different voltages (10, 20 and 30 kV) were tested. The best baseline separation was achieved using 100 mM borate buffer with 25 mM SDS at 30 kV [137]. López-Montes *et al.* used CE-DAD and CE-ESI-MS to investigate the dyes used in colour photographs such as autochromes from early 20th century. The separation condition for CE-DAD and CE-ESI-MS was at 20kV using background electrolyte (BEG) made of 50 mM ammonium acetate at pH= 9, contains 15% (v/v) acetonitrile as modifier and 60 cm capillary for CE-DAD and 62 cm for CE-ESI-MS. This study showed that CE-ESI-MS had greater sensitivity than CE-DAD. Fourteen cationic and 9 acid dyes could be identified using CE-ESI-MS [139]. In our lab, CE equipped with laser induced fluorescence (LIF) detection has been explored to study dyes and pigments. MEKC with LIF was used for the separation of selected anthraquinone and flavonoid dyes [140], [141]. Separations were carried out using 20 mM borate buffer with 20 mM SDS under alkaline conditions.

Moreover, CE has been explored to study textiles. Kouznetsov *et al.* used CE-MS for comparative study on the possible chemical modification of cellulose in eight textile samples belonging to 12th BC to 16th AD century, provided by some European museum. The separation was performed in a 70 cm capillary tube, and 100 mM borate buffer (pH= 9.0) [142]. CE can be used to study the degradation products of paper. At any stage of decomposition specific compounds such as simple carbohydrates, organic acids, and aromatic lignin derivatives are formed. Therefore, by identification of these compounds the level of

degradation could be estimated. Dupont *et al.* used CZE- UV to analyse the organic compounds originating from paper degradation [143].

It can be concluded that for the study of historical objects CE equipped with different detection schemes has been employed and the most common detectors are photo diode array (DAD), electrochemical detection (ED), mass spectrometry (MS). Relatively few studies have been carried out with fluorescence detection. The application of laser-induced-fluorescence can provide high sensitivity with very low back ground noise, and therefore a very low detection limit [144] which introduces CE -LIF as a suitable technique for studying historical objects.

1.12 Research Objectives and Thesis Organization

In conservation science, the study of dyes in museum objects is a very important task. Identifying dyes is important since, by knowing dyes the best strategic options can be chosen for conservation and restoration of the valuable objects. Regarding conservation strategies, knowledge about dyes would assist with identifying the best approaches for preservation or exhibition of museum objects. Considering restoration approaches, this information would also help the restorer to use the same colourants as those used in the original objects and choose the best restoration strategies. Although, CE equipped with DAD or MS detectors has been employed to investigate historical dyes as well as degradation products of textiles and paper, there were a few applications of capillary electrophoresis equipped with laser induced fluorescence (CE-LIF) for studying museum objects. Furthermore, one of the challenges in art conservation is to minimize the sample size for analysis to maintain the integrity of the object.

The objective of this research was to evaluate the feasibility CE-LIF as a novel technique to separate and identify dyes that could be found in textiles. Another objective of this research was to evaluate the common extraction procedures with special focus on Hanson solubility parameters.

In this research a collection of natural dyes which have been used in historical objects (textiles) was selected. These dyes were: 4 red anthraquinone dyes: alizarin, purpurin, carmine, and emodin, 8 yellow flavonoids; apigenin, luteolin, ap-7-*O*-glu, lu-7-*O*-glu (flavones), kaempferol, quercetin, morin, myricetin (flavonols), 1 yellow carotenoid dyes: crocin, 1 brown quinodic dye: lawson and 1 blue indigoid dye: indigo.

The research presented in this thesis is organized in the following Chapters (2-5):

In Chapter 2 the UV-Vis absorption and fluorescence properties of 4 dyes: alizarin, purpurin, carmine and morin were studied and the results were used towards the feasibility of separation of these dyes using MEKC-LIF. The separation was achieved using 20 mM borate buffer containing 20 mM sodium dodecyl sulfate (SDS) in a 50 cm capillary tube. The application of 2 lasers for excitation was also investigated. These lasers were a 488 nm argon ion laser (50 mW) and a 407 nm diode laser (90 mW). The results of this study were published in “Journal of Liquid Chromatography & Related Technologies” [140].

In Chapter 3 the results of separation parameters in MEKC-LIF are presented for the separation of a mixture of 4 anthraquinone dyes (alizarin, purpurin, emodin, carmine) and 6 flavonoid dyes (luteolin, apigenin, kaempferol, quercetin, morin, and myricetin), as well as a mixture of all dyes in 20 mM borate buffer with 20 mM sodium dodecyl sulfate (SDS). The separation parameters studying included the effects of pH, organic modifier (methanol

and acetonitrile) at different concentrations, and applied voltage at different values. The results of this study were published in "Chromatography" [141].

In Chapter 4 the photochemical properties of purpurin using UV-Vis and fluorescence spectrophotometry are presented. The rate constant for the photochemical fading of purpurin at different pH values were calculated. The photo-degraded products of purpurin were identified by using different analytical methods including thin layer chromatography, gas chromatography and UV-Vis spectrophotometry. The results of this chapter were published in "Dyes and Pigments" [145]. The photochemical properties of yellow flavonoid dyes kaempferol, quercetin, morin, myricetin were also studied. UV-Vis and fluorescence spectrophotometry were employed to measure the rate constant for the photochemical fading of these dyes. The magnitude of the rate constants would help to select the optimum pH for separation of dyes with CE. The results of this study were published in "Journal of Liquid Chromatography & Related Technologies" [146].

The objective of Chapter 5 is to validate the existing extraction methods with special focus on Hansen solubility parameters on the extraction of dyes from laboratory dyed samples. The laboratory samples were prepared by dyeing wool threads with madder and weld according to the historical dyeing protocol. The effects of different solvents as well as temperature and mechanical agitation at different times were investigated. Afterwards, the most successful extraction method was chosen by studying the electropherogram of each extracted dye.

In Chapter 6 the conclusion and future studies are presented.

1.13 References

1. Itten, J., *The art of color: the subjective experience and objective rationale of color*. 1993, New York: Van Nostrand Reinhold. 155.
2. Barnett, J., S. Miller, and E. Pearce, *Colour and art: A brief history of pigments*. Optics & Laser Technology, 2006. **38**(4): p. 445-453.
3. Tímár-Balázsy, Á. and D. Eastop, *Chemical principles of textile conservation*. 1998: Routledge.
4. Bechtold, T. and R. Mussak, *Handbook of natural colorants*. 2009: John Wiley & Sons.
5. Degano, I., et al., *Analytical Methods for the Characterization of Organic Dyes in Artworks and in Historical Textiles*. Applied Spectroscopy Reviews, 2009. **44**(5): p. 363-410.
6. Rodríguez, E.S., Angela Arteaga Rodríguez, María Antonia García, and Rodríguez Carmen Cámara *Characterization of Natural and Synthetic Dyes Employed in the Manufacture of Chinese Garment Pieces by C-DAD and LC-DAD-QTOF*. e-conservation, 2011/09/. **21**, 38-55.
7. Schweppe, H., *Identification of dyes on old textiles*. Journal of the American Institute for Conservation, 1979. **19**(1): p. 14-23.
8. Abel, A. and J. Best, *The history of dyes and pigments: From natural dyes to high performance pigments*. 2012, Woodhead Publishing: Cambridge. p. 433-470.
9. Maulik, S.R. and S.C. Pradhan, *Dyeing of wool and silk with hinjal bark, jujube bark and himalayan rhubarb*. Colourage, 2005. **52**(9): p. 67-71.
10. Kharbade, B. and O. Agrawal, *Analysis of natural dyes in Indian historic textiles*. Studies in conservation, 1988: p. 1-8.
11. Cardon, D., *Natural dyes: sources, tradition, technology and science*. 2007: Archetype.
12. Haslam, E., *Practical polyphenolics: from structure to molecular recognition and physiological action*. 1998: Cambridge University Press.
13. Francisco, A., *JB HARBORNE (ED.) The flavonoids—Advances in research since 1986 Chapman & Hall, London, UK 1994, £ 195.00, 676 pp. ISBN 0-412-48070-0*. Phytochemical Analysis, 1995. **6**(1): p. 55-55.
14. Rosenberg, E., *Characterisation of historical organic dyestuffs by liquid chromatography–mass spectrometry*. Analytical and bioanalytical Chemistry, 2008. **391**(1): p. 33-57.
15. Simpson, J. and E.S. Weiner, *Oxford English dictionary online*. Oxford: Clarendon Press. Retrieved March, 1989. **6**: p. 2008.
16. Ong, K.C. and H.-E. Khoo, *Biological effects of myricetin*. General Pharmacology: The Vascular System, 1997. **29**(2): p. 121-126.
17. Gulrajani, M., et al., *Some Studies on Natural Yellow Dyes, Part III: Quinones: Henna, Dolu*. The Indian Textile Journal, 1992. **102**(3): p. 76-83.
18. Agarwal, A., A. Garg, and K. Gupta, *Development of suitable dyeing process for dyeing of wool with natural dye henna (Lawsonia inermis)*. Colourage, 1992. **39**(10): p. 43-45.

19. Bechtold, T., et al., *Natural dyes in modern textile dyehouses—how to combine experiences of two centuries to meet the demands of the future?* Journal of Cleaner Production, 2003. **11**(5): p. 499-509.
20. Onal, A., N. Camci, and A. Sari, *Extraction of total dyestuff from walnut leaves (*Juglans regia* L.) and its dyeing conditions for natural fibres.* Asian Journal of Chemistry, 2004. **16**(3): p. 1533.
21. Taylor, G.W., *Detection and identification of dyes on Anglo-Scandinavian textiles.* Studies in Conservation, 1983. **28**(4): p. 153-160.
22. Nowik, W., et al., *The Analysis of Dyestuffs from First-to Second-Century Textile Artefacts Found in the Martres-de-veyre (France) Excavations.* Archaeometry, 2005. **47**(4): p. 835-848.
23. Clementi, C., et al., *A spectrometric and chromatographic approach to the study of ageing of madder (*Rubia tinctorum* L.) dyestuff on wool.* Anal Chim Acta, 2007. **596**(1): p. 46-54.
24. Schweppe, H., *Identification of dyes in historic textile materials*, in *Historic textile and paper materials: Conservation and characterization.* 1986, American Chemical Society. p. 153-174.
25. Harris, D.C., *Quantitative Chemical Analysis.* 2003: Macmillan.
26. Karadağ, R. and E. Dölen, *Examination of historical textiles with dyestuff analyses by TLC and derivative spectrophotometry.* Turkish Journal of Chemistry, 1997. **21**(2): p. 126-133.
27. Leona, M. and J. Winter, *Fiber optics reflectance spectroscopy: A unique tool for the investigation of Japanese paintings.* Studies in conservation, 2001. **46**(3): p. 153-162.
28. Leona, M., et al., *Identification of the Pre-Columbian Pigment Maya blue on Works of Art by Noninvasive UV-Vis and Raman Spectroscopic Techniques.* Journal of the American Institute for Conservation, 2013: p. 39-54.
29. Clementi, C., et al., *A spectrometric and chromatographic approach to the study of ageing of madder (*Rubia tinctorum* L.) dyestuff on wool.* Analytica chimica acta, 2007. **596**(1): p. 46-54.
30. Gulmini, M., et al., *Identification of dyestuffs in historical textiles: Strong and weak points of a non-invasive approach.* Dyes and Pigments, 2013. **98**(1): p. 136-145.
31. George, G., *General Aspects of Luminescence Spectroscopy.* Practical Fluorescence, 1990. **3**: p. 1.
32. De la Rie, E.R., *Fluorescence of paint and varnish layers (Part II).* Studies in Conservation, 1982. **27**(2): p. 65-69.
33. Wallert, A., *Unusual pigments on a Greek marble basin.* Studies in Conservation, 1995. **40**(3): p. 177-188.
34. Miliani, C., A. Romani, and G. Favaro, *A spectrophotometric and fluorimetric study of some anthraquinoid and indigoid colorants used in artistic paintings.* Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 1998. **54**(4): p. 581-588.
35. Claro, A., et al., *The use of microspectrofluorimetry for the characterization of lake pigments.* Talanta, 2008. **74**(4): p. 922-929.
36. Clementi, C., et al., *In-situ fluorimetry: A powerful non-invasive diagnostic technique for natural dyes used in artefacts: Part II. Identification of orcein and*

- indigo in Renaissance tapestries*. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 2009. **71**(5): p. 2057-2062.
37. Clementi, C., et al., *In situ fluorimetry: a powerful non-invasive diagnostic technique for natural dyes used in artefacts Part I. Spectral characterization of orcein in solution, on silk and wool laboratory-standards and a fragment of Renaissance tapestry*. Spectrochim Acta A Mol Biomol Spectrosc, 2006. **64**(4): p. 906-12.
38. Skoog, D.A., F.J. Holler, and S.R. Crouch, *Instrumental analysis*. 2007: Cengage Learning India.
39. Abrahams, D.H. and S.M. Edelstein, *A new method for the analysis of ancient dyed textiles*. American Dyestuff Reporter, 1964. **53**(1): p. 19-25.
40. Masschelein-Kleiner, L. and L. Maes. *Ancient dyeing techniques in Eastern Mediterranean regions*. in *ICOM Committee for Conservation. 5th Triennial Meeting, Zagreb, 1-8 Oct. 1978. Preprints*. 1978. International Council of Museums.
41. Low, M.J. and N.S. Baer, *Application of Infrared Fourier Transform Spectroscopy to Problems in Conservation. I. General Principles*. Studies in conservation, 1977. **22**(3): p. 116-128.
42. Shearer, J.C., et al., *FTIR in the service of art conservation*. Analytical Chemistry, 1983. **55**(8): p. 874A-880A.
43. Martoglio, P.A., et al., *Unlocking the Secrets of the Past*. Analytical chemistry, 1990. **62**(21): p. 1123A-1128A.
44. Gillard, R., et al., *The detection of dyes by FTIR microscopy*. Studies in Conservation, 1994. **39**(3): p. 187-192.
45. Colthup, N., *Introduction to infrared and Raman spectroscopy*. 2012: Elsevier.
46. Edwards, H., *Illumination of a mediaeval mystery: the FT-Raman spectroscopic analysis of red pigment from a mediaeval corbel in the church St Clement of Rome, Fiskerton*. Journal of molecular structure, 2003. **661**: p. 271-277.
47. Bruni, S., et al., *The joined use of ni spectroscopic analyses—FTIR, Raman, visible reflectance spectrometry and EDXRF—to study drawings and illuminated manuscripts*. Applied Physics A, 2008. **92**(1): p. 103-108.
48. Withnall, R., et al., *Non-destructive, in situ identification of indigo/woad and shellfish purple by Raman microscopy and visible reflectance spectroscopy*. Dyes in history and archaeology, 1992(11): p. 19-24.
49. Edwards, H.G., S.E. Jorge Villar, and K.A. Eremin, *Raman spectroscopic analysis of pigments from dynastic Egyptian funerary artefacts*. Journal of Raman spectroscopy, 2004. **35**(8-9): p. 786-795.
50. Lang, P.L., et al., *The identification of fibers by infrared and Raman microspectroscopy*. Microchemical journal, 1986. **34**(3): p. 319-331.
51. Leaver, I.H., R.E. Hester, and R.B. Girling, *Analytical Potential of Raman Spectroscopy in Wool Textile Research*. Textile Research Journal, 1988. **58**(3): p. 182-184.
52. Edwards, H., E. Newton, and J. Russ, *Raman spectroscopic analysis of pigments and substrata in prehistoric rock art*. Journal of Molecular Structure, 2000. **550**: p. 245-256.

53. Edwards, H.G.M., S.E. Jorge Villar, and K.A. Eremin, *Raman spectroscopic analysis of pigments from dynastic Egyptian funerary artefacts*. Journal of Raman Spectroscopy, 2004. **35**(89): p. 786-795.
54. Muralha, V.S., L. Burgio, and R.J. Clark, *Raman spectroscopy analysis of pigments on 16-17th c. Persian manuscripts*. Spectrochim Acta A Mol Biomol Spectrosc, 2012. **92**: p. 21-8.
55. Baran, A., et al., *In situ Raman and IR spectroscopic analysis of indigo dye*. Analytical Methods, 2010. **2**(9): p. 1372.
56. Hofenk de Graaff, J.H., W. Roelofs, and M.R.v. Bommel, *The colourful past: origins, chemistry and identification of natural dyestuffs*. 2004: Archetype publications; Abegg-Stiftung.
57. Mills, J. and R. White, *Organic chemistry of museum objects*. 2012: Routledge.
58. Guineau, B. and V. Guichard. *Identification de colorants organiques naturels par microspectrométrie Raman de résonance et par effet Raman exalté de surface (SERS)*. in *ICOM committee for conservation: 8th triennial meeting, Sydney, Australia, 6-11 September, 1987. Preprints*. 1987. The Getty Conservation Institute.
59. Centeno, S.A. and J. Shamir, *Surface enhanced Raman scattering (SERS) and FTIR characterization of the sepia melanin pigment used in works of art*. Journal of Molecular Structure, 2008. **873**(1-3): p. 149-159.
60. Ferretti, M., *Scientific investigations of works of art*. 1993: ICCROM.
61. Schreiner, M., et al., *X-rays in art and archaeology: an overview*. Powder Diffraction, 2004. **19**(01): p. 3-11.
62. Trentelman, K., et al., *The examination of works of art using in situ XRF line and area scans*. X-Ray Spectrometry, 2010. **39**(3): p. 159-166.
63. Katayama, T., et al. *Application of XRF and AMS Techniques to Textiles in the Mongol Empire*. in *MRS Proceedings*. 2007. Cambridge Univ Press.
64. Zieske, F., *An investigation of Paul Cézanne's watercolors with emphasis on emerald green*. 1995.
65. Buzzegoli, E., et al., *The use of dark pigments in Frà Bartolomeo's paintings*, in *Art et chimie, la couleur: actes du congrès*. 2000, CNRS Editions. p. 203-208.
66. NIR-EL, Y. and M. Broshi, *The red ink of the Dead Sea Scrolls*. Archaeometry, 1996. **38**(1): p. 97-102.
67. Cariveau, G.W. and M. Shelley, *A study of Rembrandt drawings using X-ray fluorescence*. Nuclear Instruments and Methods in Physics Research, 1982. **193**(1): p. 297-301.
68. Aloupi, E., A. Karydas, and T. Paradellis, *Pigment analysis of wall paintings and ceramics from Greece and Cyprus. The optimum use of X-ray spectrometry on specific archaeological issues*. X-ray Spectrometry, 2000. **29**(1): p. 18-24.
69. Masschelein-Kleiner, L., *Microanalysis of hydroxyquinones in red lakes*. Microchimica Acta, 1967. **55**(6): p. 1080-1085.
70. Daniels, V., *Further work on the dye analysis of textile fragments from Enkomi*, *Dyes Hist*. 1987, Archaeol.
71. Taylor, G. *The chemistry of madder dyeings: application of semi-quantitative TLC*. in *Dyes in History and Archaeology, 10th annual meeting, London*. 1991.

72. Scott, D.A., et al., *Technical examination of a fifteenth-century German illuminated manuscript on paper: a case study in the identification of materials*. Studies in Conservation, 2001: p. 93-108.
73. Wouters, J., *High performance liquid chromatography of anthraquinones: analysis of plant and insect extracts and dyed textiles*. Studies in Conservation, 1985. **30**(3): p. 119-128.
74. Harris, D.C., *Quantitative chemical analysis*. 2010: Macmillan.
75. Dong, M.W., *Modern HPLC for practicing scientists*. 2006: John Wiley & Sons.
76. Ploeger, R., D. Scalarone, and O. Chiantore, *The characterization of commercial artists' alkyd paints*. Journal of Cultural Heritage, 2008. **9**(4): p. 412-419.
77. Halpine, S.M., *Amino acid analysis of proteinaceous media from Cosimo Tura's 'The Annunciation with Saint Francis and Saint Louis of Toulouse'*. Studies in Conservation, 1992. **37**(1): p. 22-38.
78. Casoli, A., P. Mirti, and G. Palla, *Characterization of medieval proteinaceous painting media using gas chromatography and gas chromatography—mass spectrometry*. Fresenius' journal of analytical chemistry, 1995. **352**(3-4): p. 372-379.
79. Wallert, A. *Tannins on the parchment of the Dead Sea scrolls*. in *ICOM committee for conservation, 11th triennial meeting in Edinburgh, Scotland, 1-6 September 1996: Preprints*. 1996. James & James (Science Publishers) Ltd.
80. Pastore, P., F. Magno, and S. Volpin, *Chromatographic determination of the oxalate ion in patinas covering ancient materials*. Annali di Chimica, 1991. **81**(5-6): p. 233-241.
81. Koren, Z.C., *The unprecedented discovery of the royal purple dye on the two thousand year-old royal Masada textile*. American Institute for Conservation, The Textile Specialty Group Postprints, 1997. **7**: p. 23-34.
82. Wouters, J. and A. Verhecken, *High-performance liquid chromatography of blue and purple indigoid natural dyes*. Journal of the Society of Dyers and Colourists, 1991. **107**(7-8): p. 266-269.
83. Wouters, J., *A new method for the analysis of blue and purple dyes in textiles*. Dyes in history and archaeology, 1992. **10**: p. 17-21.
84. Wouters, J. and N. Rosario-Chirinos, *Dye analysis of pre-Columbian Peruvian textiles with high-performance liquid chromatography and diode-array detection*. Journal of the American Institute for Conservation, 1992. **31**(2): p. 237-55.
85. Yamaoka, R., et al., *The identification of dyes of Greco-Roman period fabric in Egypt using liquid chromatography/mass spectrometry*. Journal of the Mass Spectrometry Society of Japan, 1989. **37**(4): p. 249-253.
86. Ferreira, E., et al., *Development of analytical techniques for the study of natural yellow dyes in historic textiles*. Dyes in history and archaeology, 2001. **16**(17): p. 179-186.
87. Ferreira, E.S. and A. Quye, *Photo-oxidation products of quercetin and morin as markers for the characterisation of natural flavonoid yellow dyes in ancient textiles*. Dyes in history and archaeology, 2002(18): p. 63-72.
88. Szostek, B., et al., *Investigation of natural dyes occurring in historical Coptic textiles by high-performance liquid chromatography with UV–Vis and mass spectrometric detection*. Journal of Chromatography A, 2003. **1012**(2): p. 179-192.

89. Zhang, X. and R.A. Laursen, *Development of mild extraction methods for the analysis of natural dyes in textiles of historical interest using LC-diode array detector-MS*. Analytical Chemistry, 2005. **77**(7): p. 2022-2025.
90. Marques, R., et al., *Characterization of weld (*Reseda luteola* L.) and spurge flax (*Daphne gnidium* L.) by high-performance liquid chromatography-diode array detection-mass spectrometry in Arraiolos historical textiles*. J Chromatogr A, 2009. **1216**(9): p. 1395-402.
91. Miller, J.M., *Chromatography: concepts and contrasts*. 2005: John Wiley & Sons.
92. Varella, E.A., *Conservation Science for the Cultural Heritage: Applications of Instrumental Analysis*. Vol. 79. 2012: Springer Science & Business Media.
93. Colombini, M.P., et al., *Characterization of proteinaceous binders in wall painting samples by microwave-assisted acid hydrolysis and GC-MS determination of amino acids*. Studies in Conservation, 1998. **43**(1): p. 33-41.
94. Colombini, M.P., et al., *Characterisation of proteinaceous binders and drying oils in wall painting samples by gas chromatography-mass spectrometry*. Journal of Chromatography A, 1999. **846**(1): p. 113-124.
95. Colombini, M., et al., *GC-MS characterization of paint varnishes*. Microchemical Journal, 2000. **67**(1): p. 385-396.
96. Colombini, M.P., et al., *Integrated analytical techniques for the study of ancient Greek polychromy*. Talanta, 2004. **63**(4): p. 839-48.
97. Colombini, M.P., et al., *Analytical strategies for characterizing organic paint media using gas chromatography/mass spectrometry*. Accounts of chemical research, 2010. **43**(6): p. 715-727.
98. Colombini, M.P., et al., *Colour fading in textiles: A model study on the decomposition of natural dyes*. Microchemical Journal, 2007. **85**(1): p. 174-182.
99. *European Pharmacopoeia* 2005, Strasbourg. p. 74-79.
100. Jorgenson, J.W. and K.D. Lukacs, *High-resolution separations based on electrophoresis and electroosmosis*. Journal of Chromatography A, 1981. **218**: p. 209-216.
101. Kok, W., *Capillary electrophoresis: instrumentation and operation*. Vol. 4. 2013: Springer Science & Business Media.
102. Jorgenson, J.W. and K.D. Lukacs, *Capillary zone electrophoresis*. Science, 1983. **222**(4621): p. 266-272.
103. Jorgenson, J.W. and K.D. Lukacs, *Zone electrophoresis in open-tubular glass capillaries*. Analytical Chemistry, 1981. **53**(8): p. 1298-1302.
104. Kuhn, R. and S. Hoffstetter-Kuhn, *Capillary electrophoresis: principles and practice*. 2013: Springer Science & Business Media.
105. Xu, Y., *Tutorial: capillary electrophoresis*. The Chemical Educator, 1996. **1**(2): p. 1-14.
106. Breadmore, M.C., *Electrokinetic and hydrodynamic injection: making the right choice for capillary electrophoresis*. Bioanalysis, 2009. **1**(5): p. 889-894.
107. Weinberger, R., *Practical capillary electrophoresis*. 2000: Academic Press.
108. Landers, J.P., *Handbook of capillary and microchip electrophoresis and associated microtechniques*. 2007: CRC press.
109. Dolník, V., S. Liu, and S. Jovanovich, *Capillary electrophoresis on microchip*. Electrophoresis, 2000. **21**(1): p. 41-54.

110. Svec, F. and Z. Deyl, *Capillary electrochromatography*. Vol. 62. 2001: Elsevier.
111. Colón, L.A., *Joule heating in packed capillaries used in capillary electrochromatography*. Electrophoresis, 2002. **23**: p. 2918-2928.
112. Whatley, H., *Basic principles and modes of capillary electrophoresis*, in *Clinical and Forensic Applications of Capillary Electrophoresis*. 2001, Springer. p. 21-58.
113. Dziubakiewicz, E. and B. Buszewski, *Principles of Electromigration Techniques*, in *Electromigration Techniques*. 2013, Springer. p. 5-26.
114. Terabe, S., et al., *Electrokinetic separations with micellar solutions and open-tubular capillaries*. Analytical Chemistry, 1984. **56**(1): p. 111-113.
115. Hancu, G., et al., *Principles of micellar electrokinetic capillary chromatography applied in pharmaceutical analysis*. Adv Pharm Bull, 2013. **3**(1): p. 1-8.
116. Gröbl, M., et al., *Characterisation of natural polysaccharides (plant gums) used as binding media for artistic and historic works by capillary zone electrophoresis*. Journal of Chromatography A, 2005. **1077**(1): p. 80-89.
117. Surowiec, I., I. Kaml, and E. Kenndler, *Analysis of drying oils used as binding media for objects of art by capillary electrophoresis with indirect UV and conductivity detection*. Journal of Chromatography A, 2004. **1024**(1-2): p. 245-254.
118. Kaml, I., K. Vcelakova, and E. Kenndler, *Characterisation and identification of proteinaceous binding media (animal glues) from their amino acid profile by capillary zone electrophoresis*. J Sep Sci, 2004. **27**(3): p. 161-6.
119. Harrison, S.M., et al., *Identification of drying oils in mixtures of natural binding media used for artistic and historic works by capillary electrophoresis*. Journal of Separation Science, 2005. **28**(13): p. 1587-1594.
120. Harrison, S.M., et al., *Animal glues in mixtures of natural binding media used in artistic and historic objects: identification by capillary zone electrophoresis*. Analytical and bioanalytical chemistry, 2005. **382**(7): p. 1520-1526.
121. Findeisen, A., et al., *Analysis of diterpenoic compounds in natural resins applied as binders in museum objects by capillary electrophoresis*. J Chromatogr A, 2007. **1157**(1-2): p. 454-61.
122. Song-gang, J., et al., *Separation and determination of anthraquinone derivatives in rhubarb and its preparations by micellar electrokinetic capillary chromatography*. Biomedical Chromatography, 1998. **12**(6): p. 335-337.
123. Weng, W.C. and S.J. Sheu, *Separation of Anthraquinones by Capillary Electrophoresis and High-Performance Liquid Chromatography*. Journal of High Resolution Chromatography, 2000. **23**(2): p. 143-148.
124. Watanabe, T. and S. Terabe, *Analysis of natural food pigments by capillary electrophoresis*. Journal of Chromatography A, 2000. **880**(1): p. 311-322.
125. Tian, K., et al., *Determination of five anthraquinones in medicinal plants by capillary zone electrophoresis with beta-cyclodextrin addition*. J Chromatogr A, 2006. **1123**(1): p. 134-7.
126. Shang, X. and Z. Yuan, *Determination of active components in Rhubarb and study of their hydrophobicity by micellar electrokinetic chromatography*. Bioorganic & Medicinal Chemistry Letters, 2003. **13**(4): p. 617-622.
127. Li, F., Q.-E. Cao, and Z. Ding, *Separation and determination of five anthraquinones in rheum and rheum-containing preparations by capillary zone electrophoresis*. Chromatographia, 2004. **59**(11-12): p. 753-757.

128. Jiang, T.-F., Z.-H. Lv, and Y.-H. Wang, *Separation and determination of anthraquinones in Cassia obtusifolia (Leguminosae) by micellar electrokinetic capillary electrophoresis*. Journal of Separation Science, 2005. **28**(16): p. 2225-2229.
129. Koyama, J., et al., *Simultaneous Determination of Anthraquinones, Their 8-BETA.-D-Glucosides, and Sennosides of Rhei Rhizoma by Capillary Electrophoresis*. Chemical and pharmaceutical bulletin, 2005. **53**(5): p. 573-575.
130. Sun, S.-W. and P.-C. Yeh, *Analysis of rhubarb anthraquinones and bianthrone by microemulsion electrokinetic chromatography*. Journal of pharmaceutical and biomedical analysis, 2005. **36**(5): p. 995-1001.
131. Maguregui, M.I., et al., *Micellar electrokinetic chromatography method for the determination of several natural red dyestuff and lake pigments used in art work*. J Chromatogr A, 2007. **1154**(1-2): p. 429-36.
132. Svobodová, E., et al., *Use of Capillary Zone Electrophoresis and Micellar Electrokinetic Chromatography for Separations of Anthraquinone Derivatives*. Analytical Letters, 2011. **44**(10): p. 1783-1795.
133. Farry, L., et al., *A study of the effects of injection mode, on-capillary stacking and off-line concentration on the capillary electrophoresis limits of detection for four structural types of industrial dyes*. Analytica chimica acta, 1997. **349**(1): p. 221-229.
134. Sirén, H. and R. Sulkava, *Determination of black dyes from cotton and wool fibres by capillary zone electrophoresis with UV detection: application of marker technique*. Journal of Chromatography A, 1995. **717**(1): p. 149-155.
135. Poiger, T., S.D. Richardson, and G.L. Baughman, *Analysis of anionic metallized azo and formazan dyes by capillary electrophoresis-mass spectrometry*. Journal of Chromatography A, 2000. **886**(1): p. 259-270.
136. Zhao, X., et al., *Study of biodegradation products from azo dyes in fungal degradation by capillary electrophoresis/electrospray mass spectrometry*. J Chromatogr A, 2007. **1159**(1-2): p. 217-24.
137. Zlotnick, J. and F. Smith, *Separation of some black rollerball pen inks by capillary electrophoresis: preliminary data*. Forensic Science International, 1998. **92**(2): p. 269-280.
138. Vogt, C., et al., *Separation, comparison and identification of fountain pen inks by capillary electrophoresis with UV-visible and fluorescence detection and by proton-induced X-ray emission*. Journal of Chromatography A, 1997. **781**(1): p. 391-405.
139. López-Montes, A.M., et al., *Identification of synthetic dyes in early colour photographs using capillary electrophoresis and electrospray ionisation-mass spectrometry*. Talanta, 2013. **114**: p. 217-226.
140. Goltz, D.M., et al., *Separation of historical dyes using capillary electrophoresis with laser-induced fluorescence detection*. Journal of Liquid Chromatography & Related Technologies, 2012. **35**(14): p. 2054-2065.
141. Ahmadi, S., D.B. Craig, and D.M. Goltz, *Micellar electrokinetic chromatography with laser-induced fluorescence detection for separation of red and yellow historical dyes*. Chromatography, 2013. **1**(1): p. 9-23.

142. Kouznetsov, D.A., A.A. Ivanov, and P.R. Veletsky, *Detection of alkylated cellulose derivatives in several archaeological linen textile samples by capillary electrophoresis/mass spectrometry*. Analytical chemistry, 1994. **66**(23): p. 4359-4365.
143. Dupont, A.-L., et al., *Comprehensive characterisation of cellulose-and lignocellulose-degradation products in aged papers: Capillary zone electrophoresis of low-molar mass organic acids, carbohydrates, and aromatic lignin derivatives*. Carbohydrate Polymers, 2007. **68**(1): p. 1-16.
144. Craig, D., et al., *Fluorescence-based enzymatic assay by capillary electrophoresis laser-induced fluorescence detection for the determination of a few β -galactosidase molecules*. Analytical biochemistry, 1995. **226**(1): p. 147-153.
145. Ahmadi, S., et al., *Photochemical properties of purpurin and its implications for capillary electrophoresis with laser induced fluorescence detection*. Dyes and Pigments, 2014. **105**: p. 57-62.
146. Goltz, D., et al., *Photochemical properties of selected flavonol dyes: Effects on their separation using capillary electrophoresis*. Journal of Liquid Chromatography & Related Technologies, 2016. **39**(16): p. 768-774.

Chapter 2

Feasibility study of the application of
CE-LIF for identification of selected
historical dyes

Separation of Historical Dyes Capillary Electrophoresis with Laser-Induced Fluorescence Detection

Douglas M. Goltz ^{1,2}, Shokoufeh Ahmadi², Ghodrattollah Absalan³ and Douglas B. Craig¹

1. Centre for Scientific and Curatorial Analysis of Painting Elements (C-SCAPE)
Department of Chemistry, University of Winnipeg, 515 Portage Ave., Winnipeg,
MB, R3B 2E9, CANADA
2. Department of Chemistry, University of Manitoba, Winnipeg Manitoba, CAN-
ADA
3. Department of Chemistry, Shiraz University, Shiraz, Iran

Individual contribution of authors:

Shokoufeh Ahmadi performed all analysis/separations, data collection, data interpretation as well as drafted the manuscript for publication.

Dr. Doug Goltz supervised the development of experiments and wrote sections of the introduction/discussion of the manuscript and was the corresponding author.

Dr. Doug Craig made a contribution experimentally and helped edit the manuscript prior to publication.

Dr. Ghodrattollah Absalan critically reviewed the manuscript and helped edit portions of the manuscript prior to publication.

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2.1 Introduction

Capillary electrophoresis equipped with UV-Vis spectroscopic detector (DAD: diode array detector) has been mostly used in the field of art conservation to identify binding media, adhesives, coatings varnishes and pigments in paintings. The application of CE with different detection schemes has been previously investigated to study natural and synthetic dyes in textiles [1-11]. According to literature, micellar electrokinetic chromatography (MEKC) mode is the most popular for separation of natural and synthetic dyes. For the separation of anthraquinones and flavonoids in Coptic textiles from the National Museum in Warsaw (Poland) MEKC has been described [12]. In this study, a mixture of dyes was separated using borate buffer at pH= 8.5. One of the yellow samples was dyed with weld and madder due to identification of alizarin, purpurin, luteolin, apigenin. The peaks assigned for alizarin, purpurin and apigenin in the electropherogram were Gaussian as opposed to luteolin peak which can hardly be considered as a “peak”. The orange sample was analysed and 2 small peaks were assigned for purpurin and luteolin that are mostly similar to baseline noise [12]. López-Montes *et al.* used CE in the MEKC mode equipped with UV to study the natural dyes from a collection of drawing and maps from the Royal Chancellery Archives in Granada. A symmetrical peak was identified as carminic acid in the red sample. Two yellow samples were studied and a very small peak for gambogic acid could be detected in one of the yellow samples. In another sample crocetin and gambogic acid were detected. This sample was analysed with HPLC-DAD and the same results were achieved, in a longer run time (14 minutes vs 42 minutes) with a poor peak shape for crocetin. The separation was achieved in a 64.5 cm capillary test tube, using a 20 mM borate buffer (pH=

9.25) [13]. This research group used CE-UV to study the sepia ink in documents and maps belong to 16th -19th centuries. The CE parameters were investigated and it showed that the resolution of peaks was related to freshness of sample since sepia ink lost its stability after 24 hours. The best peak resolution was achieved at pH= 9.2 using borate buffer [14]. Puchalska used CE coupled with diode-array detection (DAD) and electrospray mass spectrometric (ESI-MS) detection to isolate and identify the colourants of some anthraquinone dyes with animal and plant origin. The separation conditions for CE-DAD was 55 cm capillary, a background electrolyte (BGE) containing 5 mM sodium phosphate (pH= 8.5), and for CE-ESI-MS the capillary was 85 cm and the BEG was 20 mM ammonium carbonate (pH= 9.0). By using CE-DAD alizarin and purpurin were detected in madder, laccaic acid in lac dye and carminic acid in cochineal. The application of CE-ESI-MS showed better selectivity and sensitivity than CE-DAD, while it allowed detection of different laccaic acid isomers known as A, B, and E in the sample [15]. The separation of anthraquinones and flavonoids using CE with MS detection has also been described by Surowiec *et al.* They studied the influence of CE parameters to achieve the optimum baseline separation for a mixture of red and yellow dyes including rhamnetin, quercetin, apigenin, luteolin, emodin, xanthopurpurin, morin, alizarin, gallic acid, purpurin, and carminic acid. The influence of buffer concentration and pH on dyes migration time and peak resolution were investigated. By changing the concentration of buffer from 20 mM to 40 mM the run time for all dyes increased except for gallic acid which remained unchanged. Moreover, flavonoids showed better separation efficiency at higher concentration. The migration time for all dyes showed a sharp decline when pH changed from 9 to 9.5. However, when pH

changed to 10 most of the dyes showed steady decreasing trends except purpurin, xanthopurpurin, carminic acid and gallic acid. Baseline separation was achieved using 40 mM ammonium acetate at pH= 9.5 using 25 kV. It seems the peaks assigned for rhamnetin, apigenin, and purpurin were not well resolved [16].

From the published literature, it can be concluded that mostly CE equipped with UV-Vis light source has been used to study the artifacts. The application of laser can provide the lowest detection limit, therefore CE-equipped with laser source would have an advantage over CE equipped with UV-Vis light source. The laser beam is very narrow, with minimal divergence and the ability to be focused between the inner walls as well as end-tip of the capillary tube [17]. The laser beam also produces very intense fluorescence, which can be detected by photo multiplier tube (PMT) with a low detection limit of (1 nM- 1 μ M) [18]. The lowest detection limit can also be attained by using optical filters in front of the PMT which can block the scattering of the laser beam. Moreover, using a RC-filter (resistor-capacitor circuit) by reducing noise and electrical interference can further improve the detection limit [17]. The most popular lasers equipped with CE are: HeNe (543 and 632 nm), Ar⁺ (488 and 514 nm) and diode (645 and 840 nm). Therefore, due to having very narrow beams, with minimal divergence and the ability to be focused even low energy output lasers such as HeNe laser may be used for excitation of eluent [17]. The requirement that samples possess a chromophore, which fluoresces at a specific wavelength can be a disadvantage using CE equipped with laser. Therefore, CE-LIF can be a preferable method for those dyes, which can fluoresce at the specific wavelength of laser equipped to CE. Due to acquiring the minimum amount of historical samples that can be sacrificed for analysis, CE-LIF would be a suitable technique for the dyes which fluoresce at the laser wavelength.

In this research, CE in the mode of (MEKC) equipped with laser induced fluorescence has been employed. **Figure 2.1** depicts the CE-LIF instrumentation which consists of the following parts: a fused silica capillary tube, a high voltage power supply, a sheath flow cuvette, numerical aperture microscope objectives, optical filter, a PMT detector and a computer.

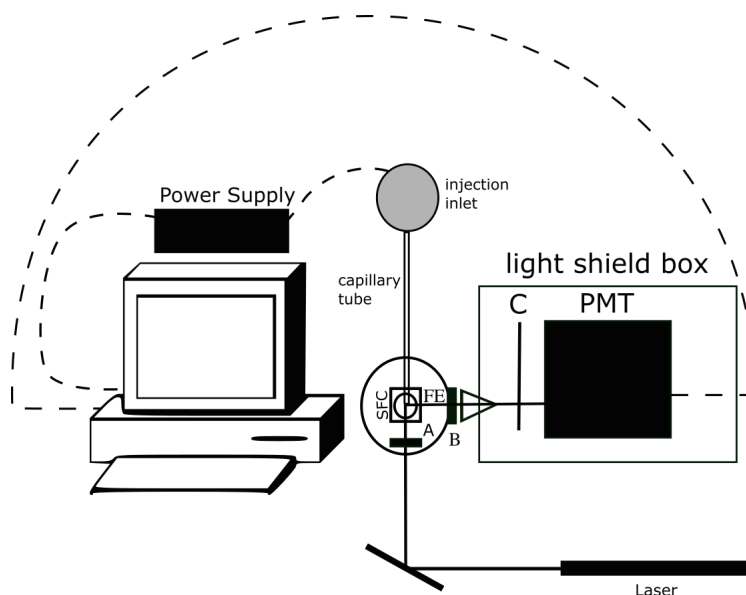


Figure 2.1 The CE-LIF instrumentation diagram: A: 6.3X 0.2 N.A. microscope objective, SFC: sheath flow cuvette, B: 60X 0.7 N.A. microscope objective, C: optical filter, FE: fluorescence emission.

The objective of research presented in this chapter was to determine if CE-LIF could be applied for separation and identification of a selection of historical dyes. In CE-LIF the identification of dyes is based on their fluorescence properties which is affected by the wavelength of laser source equipped with CE. Therefore, in order to choose the suitable available laser source which can be used for the dyes under this study, the fluorescence property of each dye was initially examined using fluorimeter. CE separation properties

are influenced by concentration and pH of buffer, type and concentration of modifier, and applied voltage. Therefore, the optimum condition for baseline separation of a small number of dyes under this study was defined by manipulating all these factors prior to separating larger number of dyes.

2.2 Experimental

Separations were performed using a laboratory-constructed CE instrument equipped with a post-column laser-induced fluorescence detection system [19]. The injection end of a 50 cm long, 10 μm inner diameter fused silica capillary (Polymicro Technologies), along with a platinum electrode connected to a high voltage supply (Spellman model CZE 2000), was placed into a buffer-filled vessel. The applied voltage across the capillary was typically 16 kV. The detection end of the capillary, from which a 1 mm length of the external polyimide coating was removed by flame, was inserted into a quartz sheath flow cuvette with a 250 by 250 μm inner bore (Hellma). The system was grounded through the sheath flow buffer within the cuvette. Two laser sources were employed: an argon ion laser (Melles Griot) tuned to 488 nm (50 mW) and a diode laser (Coherent) with a 407 nm output (90 mW). Light from the laser was focused with a 6.3 X, N.A. 0.2 microscope objective (Melles Griot) approximately 10 μm below the detection end of the capillary. Emission was collected at 90° using a 60 X, N.A. 0.7 microscope objective (Universe Kogaku), passed through a 535Df35 optical filter (Omega Optical) and a slit and onto a PMT (Hamamatsu model 1477). The analog PMT signal was collected and digitized using a Pentium 4 com-

puter through a PCI-MIO-16XE I/O board utilizing LabView software (National Instruments) at 10 Hz. The same board was used to control the electrophoresis voltage and PMT bias.

A bench top Varian Cary eclipse fluorescence spectrophotometer was used to measure the excitation and emission spectra of the dyes. Measurements were carried out using a quartz cell with a 1 cm pathlength. The excitation and emission slits were set to 5 nm and in addition to absorption spectra, emission spectra were also measured at a fixed excitation wavelength of either 407 nm or 488 nm.

2.3 Reagents

Dimethyl sulfoxide (DMSO) and acetonitrile were obtained from Sigma-Aldrich. Methanol and isopropyl alcohol were purchased from EMD Chemicals Inc., sodium dodecyl sulfate (SDS), 99% and sodium hydroxide NaOH were purchased from Sigma-Aldrich. Sodium borate was purchased from Mallinckrodt. Alizarin and carmine were purchased from Alfa Aesar. Purpurin and morin were from Fluka/Sigma-Aldrich. Fluorescein was from Molecular Probes. The CE buffer typically consisted of 20 mM sodium borate buffer pH = 9.2 and 20 mM sodium dodecyl sulfate (SDS) in Milli-Q water (18 M Ω ·cm resistance). Standard stock solutions of alizarin, purpurin, carmine and morin (1000 $\mu\text{g ml}^{-1}$) were prepared by dissolving 10 mg of powder in 10 ml dimethyl sulfoxide (DMSO) and diluted as required to 0.5-10 $\mu\text{g ml}^{-1}$ with buffer.

2.4 Discussion

The baseline separation can be achieved by modifying the following parameters: the concentration of buffer as well as its pH, the application of modifier and applied voltage.

In this feasibility study of CE these parameters were investigated by separation of 3 anthraquinone dyes: alizarin, purpurin and carmine and one flavonoid dye (morin).

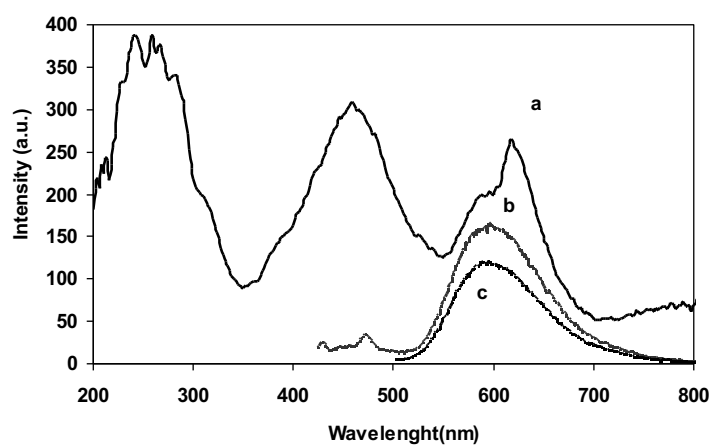
Laser sources that were available in the lab and could be coupled with CE were Ar 488 nm; 50 mw, and diode laser 407 nm, 90 mw.

In order to choose a suitable laser wavelength, fluorescence properties of $10\ \mu\text{g ml}^{-1}$ solution of each dye in 20 mM borate buffer pH= 9.2, were investigated using a bench top fluorescence spectrophotometer. Since the available laser sources were at 488 nm, and 407 nm, therefore, the emission spectrum of each dye was collected using these 2 excitation wavelengths.

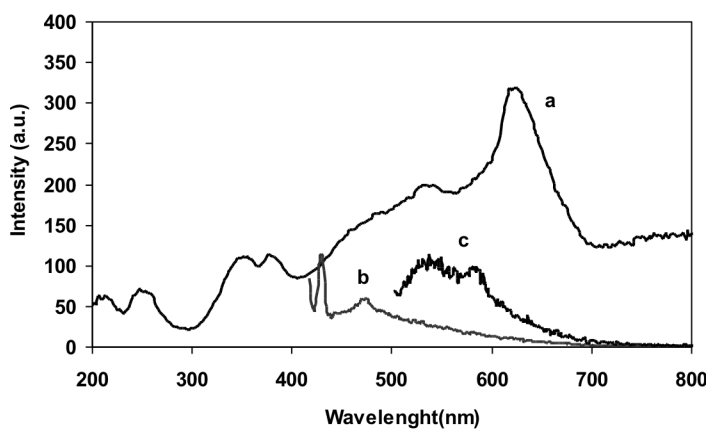
The absorption and emission spectra of alizarin, purpurin, carmine and morin are shown in **Figures 2.2 (a-d)**. The emission spectra of the dyes were acquired using 2 wavelengths for excitation: 407 nm and 488 nm. These wavelengths were chosen, as they were consistent with the lasers used for excitation in the CE-LIF instrument. For 3 of the dyes the excitation wavelength affected the maximum emission wavelength. The exception was alizarin whereby the excitation wavelength did not significantly affect the maximum emission wavelength.

In general, absorption peaks for alizarin were much larger than those of purpurin, particularly in the ultraviolet. For alizarin the emission spectra was not significantly affected

by the wavelength of the excitation source. The emission spectra for purpurin, carmine and morin had peaks at longer wavelengths when they were excited at a longer wavelength. Overall, these spectra suggest that for fluorescence based detectors, alizarin and morin would likely be measured with the highest sensitivity as compared to purpurin and carmine because the intensity of emitted light for alizarin and morin is greater than those of purpurin and carmine.



(a)



(b)

Figure 2.2 a Absorption (a) and emission spectra of alizarin (b) 407 nm source (c) 488 nm source, **Figure 2.2 b** Absorption (a) and emission spectra of purpurin (b) 407 nm source (c) 488 nm source. Adapted from Ref. [20] – Copyright: Taylor and Francis.

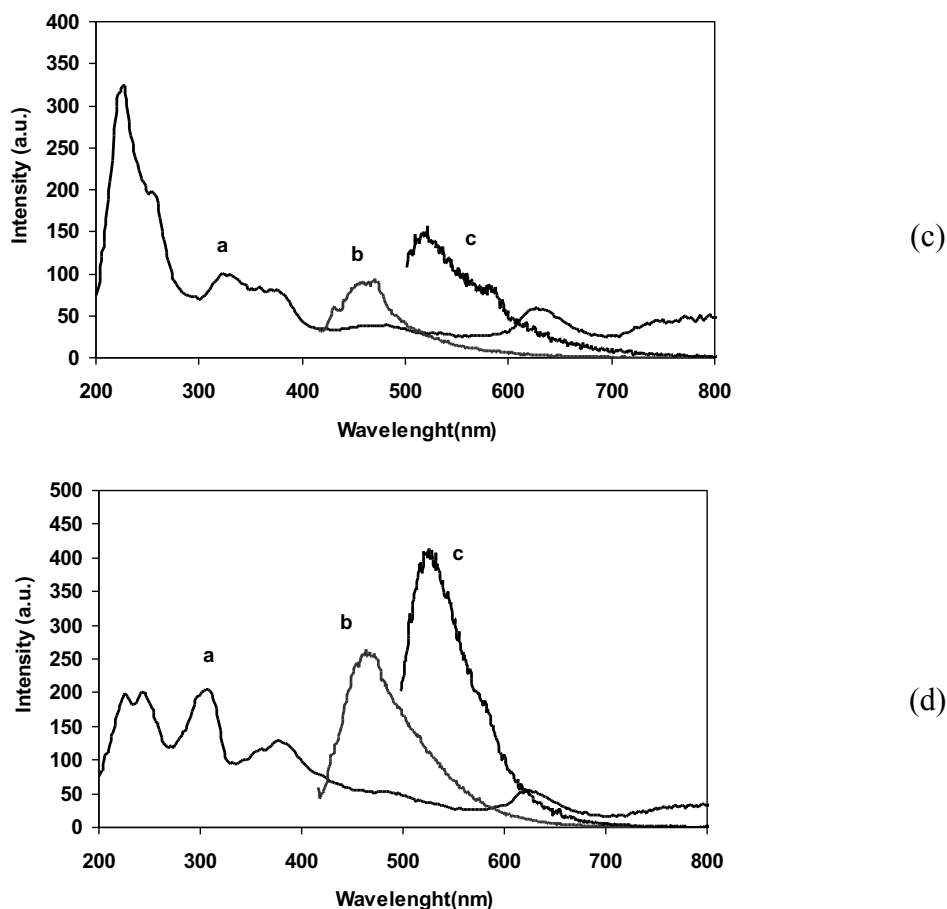


Figure 2.2 c Absorption (a) and emission spectra of carmine (b) 407 nm source (c) 488 nm source. **Figure 2.2 d** Absorption (a) and emission spectra of morin (b) 407 nm source (c) 488 nm source. Adapted from Ref. [20] – Copyright: Taylor and Francis.

The acquisition of fluorescence emission spectra was also useful for assessing the appropriate detection wavelength. The detection wavelength was selected using an interference filter. The interference filter is also useful for removing scattered light. Because different dyes have different spectral properties, compromise conditions are required for detecting them all at the same wavelength.

Alizarin, carmine, morin and purpurin were each prepared individually in $10 \mu\text{g ml}^{-1}$ concentrations. Each dye ran individually through a $10 \mu\text{m}$ capillary at 16 kV in a 20 mM

borate and 20 mM SDS buffer, pH= 9.2. The dyes were run constantly through the capillary. The filter in front of the PMT was changed, the signal and background were recorded, then the next filter was placed into the PMT. These measurements were done with lights off so that the light would not contribute to the background signal. The results from this study are summarised in **Table 2.2**. From **Table 2.2** it can be concluded that the 580 nm filter transmitted visible light, which caused the detector to produce very large background signals, so much so that when the electric field was turned on, the signal was actually lower than with no dye flowing through the capillary.

Table 2.1 The results of measuring of average of 3 readings of fluorescence intensity (FI), the background noise, the ratio of signal to noise (S/N), and the fluorescence intensity minus the background noise (SNR) for alizarin, carmine, morin and purpurin using different filters in front of PMT

Alizarin				
Filter (nm)	FI (n=3)	Background	S/N	SNR
500 ± 40	0.67 ± 0.03	0.4	1.68	0.27
510 ± 23	1.01 ± 0.02	0.4	2.52	0.61
535 ± 46	1.70 ± 0.02	0.49	3.46	1.21
580 ± 40	7.28 ± 0.17	7.27	1.00	0.01
615 ± 45	3.05 ± 0.07	0.41	7.44	2.64
630 ± 50	2.12 ± 0.05	0.35	6.06	1.77

Table 2.2 **continued**

Purpurin				
Filter (nm)	FI (n=3)	Background	S/N	SNR
500 ± 40	0.51 ± 0.01	0.33	1.56	0.18
510 ± 23	0.72 ± 0.01	0.37	1.95	0.35
535 ± 46	1.39 ± 0.02	0.47	2.95	0.92
580 ± 40	0.00	9.76	0.00	-9.76
615 ± 45	1.12 ± 0.03	0.32	3.50	0.80
630 ± 50	0.84 ± 0.04	0.22	3.82	0.62
Carmine				
Filter (nm)	FI (n=3)	Background	S/N	SNR
500 ± 40	0.19 ± 0.00	0.16	1.16	0.03
510 ± 23	0.25 ± 0.01	0.18	1.37	0.07
535 ± 46	0.39 ± 0.02	0.23	1.71	0.16
580 ± 40	3.61 ± 0.04	3.65	0.99	-0.04
615 ± 45	0.43 ± 0.02	0.18	2.37	0.25
630 ± 50	0.35 ± 0.01	0.11	3.15	0.24

Table 2.2 **continued**

Morin				
Filter (nm)	FI (n=3)	Background	S/N	SNR
500 ± 40	2.10 ± 0.03	0.32	6.55	1.78
510 ± 23	4.42 ± 0.05	0.35	12.62	4.07
535 ± 46	8.05 ± 0.10	0.41	19.64	7.64
580 ± 40	2.06 ± 0.04	1.99	1.04	0.07
615 ± 45	2.55 ± 0.06	0.13	19.64	2.42
630 ± 50	1.95 ± 0.11	0.11	17.73	1.84

The fluorescent background signal detected as the dye passed through the cuvette produced a large signal, and therefore signal from the dye itself went undetected.

The signals produced when using different filters were compared to find which signal was the most appropriate for the dyes in this separation by comparing the signal to noise ratio of each filter, to figure out which signal was actually the largest. It was found that the 535 nm filter had the most consistently large signal and less background noise.

2.4.1 Evaluation of laser sources in CE-LIF

The excitation wavelength was also evaluated using 2 different lasers. For this experiment the 407 nm and 488 nm lasers light sources were evaluated for the detection of a mixture of 4 dyes alizarin, purpurin, carmine and morin. **Figure 2.3**, illustrates the application of 2 laser sources for separation of these dyes. It appears that both lasers are useful as excitation sources. For the detection of alizarin the 407 nm laser appears to give larger emission

peaks, which provides a higher sensitivity for this particular dye. Certainly with the limited number of dyes used in this experiment, it is difficult to assume that all flavonoid or anthraquinone dyes could be determined equally well.

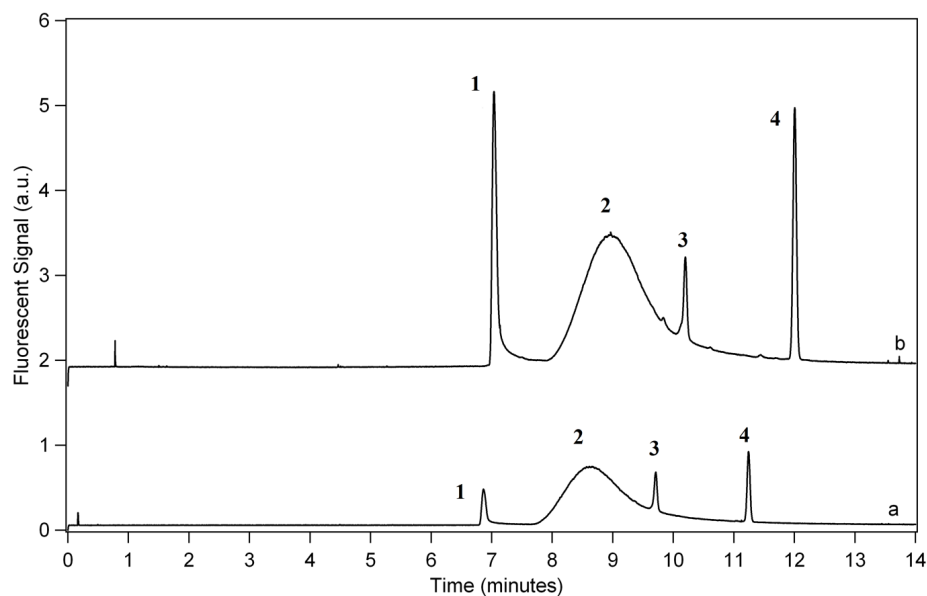


Figure 2.3 Separation of (1) $5 \mu\text{g ml}^{-1}$ alizarin, (2) $5 \mu\text{g ml}^{-1}$ purpurin, (3) $10 \mu\text{g ml}^{-1}$ carmine and (4) $0.5 \mu\text{g ml}^{-1}$ morin using 2 lasers as excitation sources (50 mW): **(a)** 488 nm and **(b)** 407 nm, using borate buffer 20 mM with 20 mM SDS, at 20 kV.

2.4.2 Dissolution of dyes

The first approach was dissolving dyes in a proper solvent. In a preliminary experiment, the stock solutions of dyes were prepared by using background electrolyte which was a 20 mM borate buffer at pH= 9.2 with 20 mM SDS. Among 4 dyes morin was the only dye that dissolved completely in this solvent. The solubility of these dyes was investigated in various solvents with different polarities such as ethanol, acetone, methanol, acetonitrile, dimethyl sulfoxide (DMSO) and it was found that DMSO was the only solvent that could properly dissolve these dyes. By comparing the polarity (I) of these solvents: ethanol= 4.3,

acetone, methanol= 5.1, acetonitrile= 5.8, and DMSO= 7.2 [21] it was concluded that these natural dyes could be dissolved properly in the more polar solvent.

Therefore, DMSO was used as a solvent for all the stock solutions of these dyes, which means that DMSO is present in low concentrations in all the diluted dye solutions. To study the effect of this solvent, 2 concentrations of DMSO were used (2.5% v/v and 7.5% v/v) in a 20 mM borate buffer pH= 9.2 for the separation of alizarin, purpurin, carmine and morin. The amount of DMSO should not be more than the amount, which affects the micelles aggregation number (N_g).

As shown in **Figure 2.4**, a small increase in concentration of DMSO increased the viscosity of the BGE, which in turn decreased the mobility of the eluents and resulted in a longer migration time.

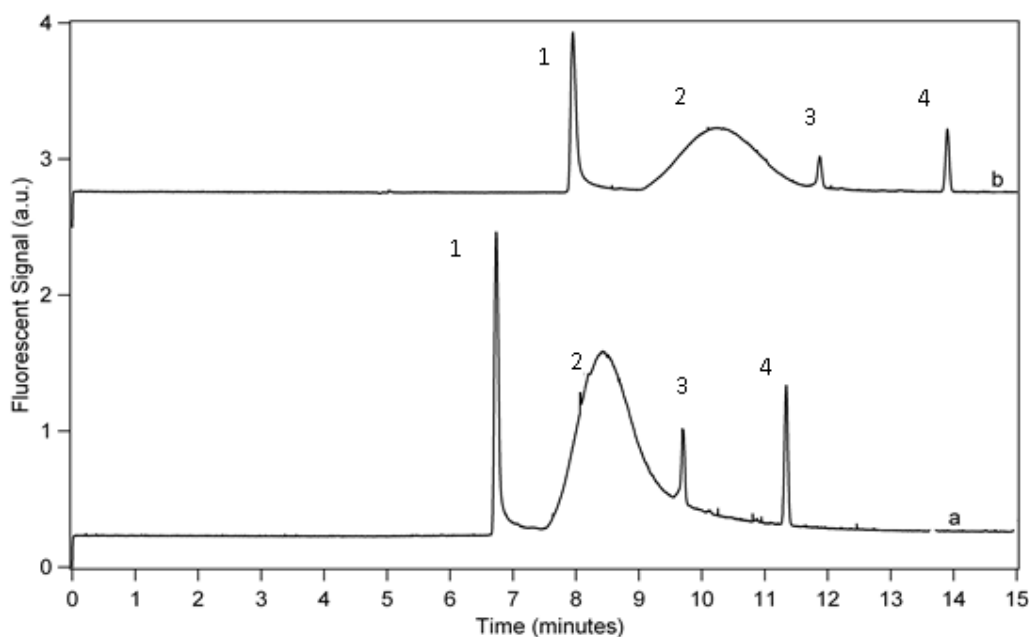


Figure 2.4 Separation of (1) $5 \mu\text{g ml}^{-1}$ alizarin, (2) $5 \mu\text{g ml}^{-1}$ purpurin, (3) $10 \mu\text{g ml}^{-1}$ carmine and (4) $0.5 \mu\text{g ml}^{-1}$ morin using borate buffer 20 mM with 20 mM SDS, at 20 kV. (a) 2.5% (12.5 μl) DMSO in 20 mM borate buffer, (b) (7.5%) 37.5 μl of DMSO in 20 mM borate. Adapted from Ref. [20] – Copyright: Taylor and Francis.

2.4.3 Buffer concentration

The concentration of buffer has an impact on EOF and mobility of the analytes. For most separations, buffer concentration was from 10 to 50 mM, therefore the effect of concentration of borate buffer at pH= 9.2 with 20 mM SDS at 10, 20, 30 and 50 mM was investigated.

The results are given in **Figure 2.5**.

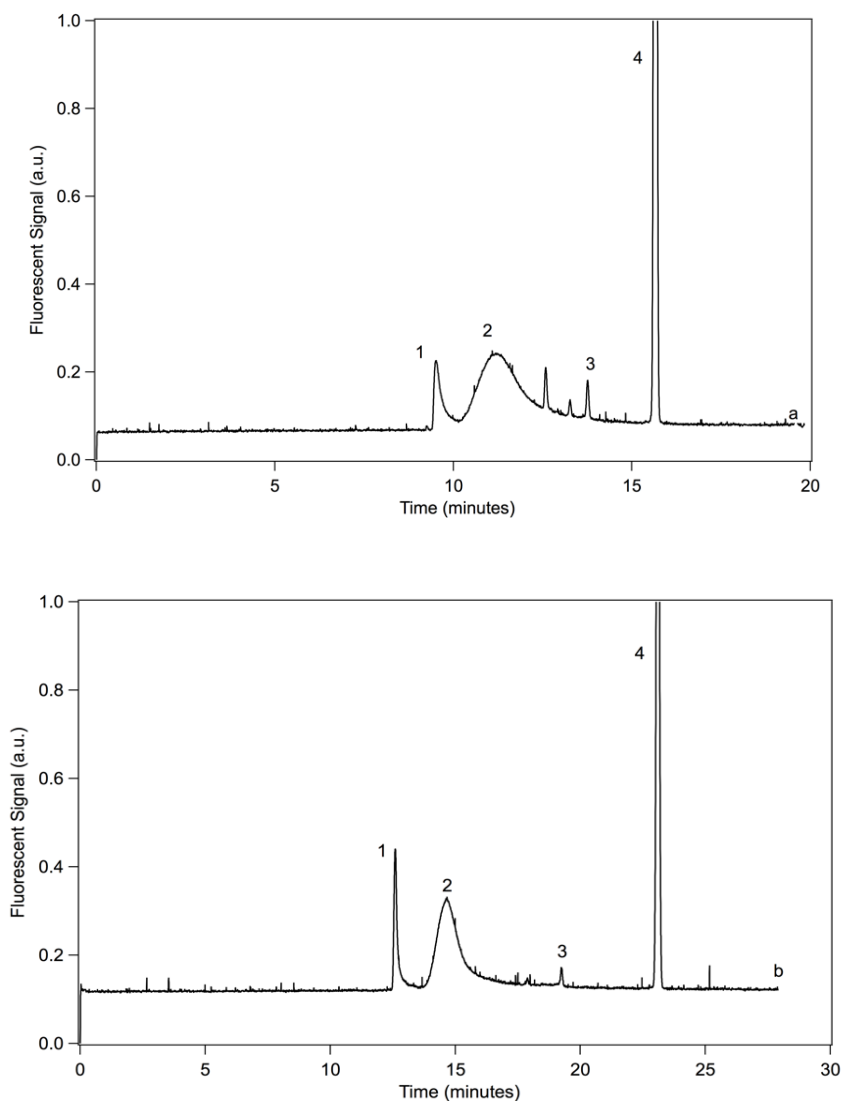


Figure 2.5 a, b Separation of (1) $5 \mu\text{g ml}^{-1}$ alizarin, (2) $10 \mu\text{g ml}^{-1}$ purpurin, (3) $10 \mu\text{g ml}^{-1}$ carmine and (4) $5 \mu\text{g ml}^{-1}$ morin at 16 kV, using different concentration of borate buffer at pH= 9.2 with 20 mM. SDS (**a**) 10 mM, (**b**) 20 mM.

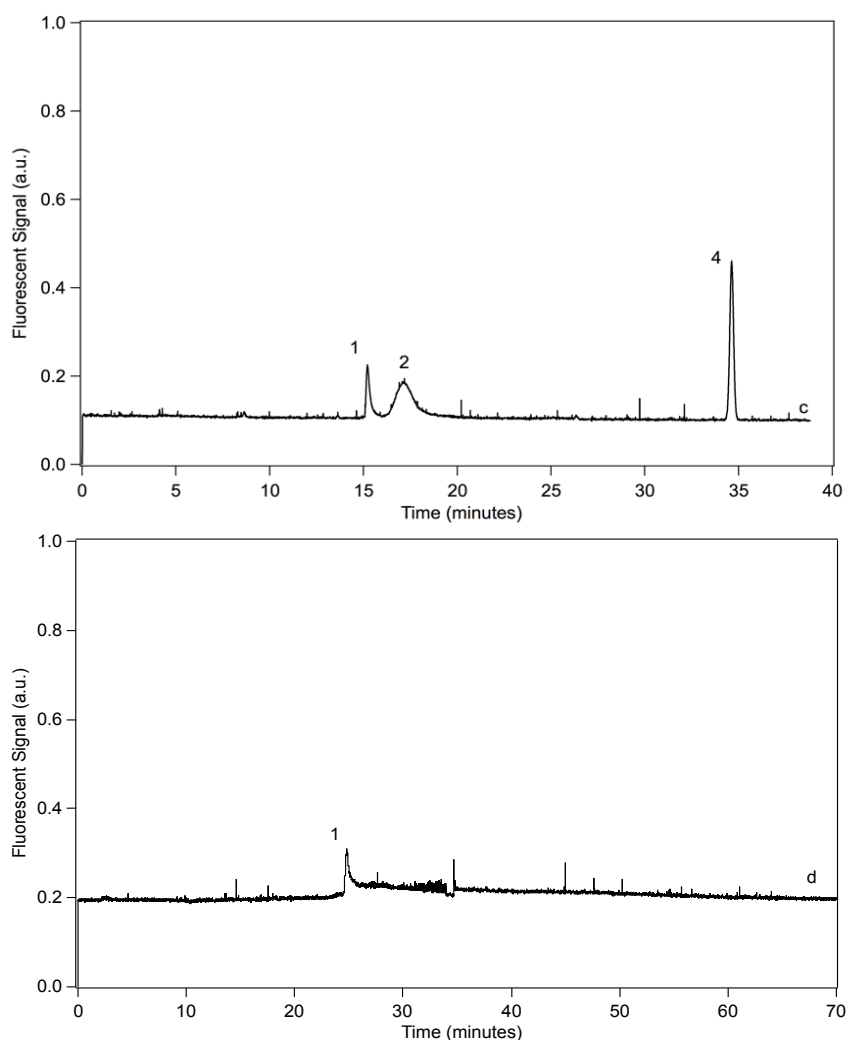


Figure 2.5 c, d Separation of (1) 5 $\mu\text{g ml}^{-1}$ alizarin, (2) 10 $\mu\text{g ml}^{-1}$ purpurin, (3) 10 $\mu\text{g ml}^{-1}$ carmine and (4) 5 $\mu\text{g ml}^{-1}$ morin at 16 kV, using different concentration of borate buffer at pH= 9.2 with 20 mM SDS (c) 30 mM. and (d) 50 mM .

In **Figure 2.5-a**, the 4 dyes were separated from each other at 10 mM concentration in 16 minutes. The peak shapes of most dyes are reasonable except for purpurin, which is quite broad. By increasing the concentration to 20 mM, the dyes separated in 24 minutes and alizarin and purpurin showed sharper peaks with less broadening while carmine yielded a smaller peak. In 30 mM (**Figure 2.5-c**), the separation was achieved in 35

minutes. The carmine peak was too small to be detected. Regarding other dyes their peak shapes were the same as ones in 20 mM with smaller size.

As **Figure 2.5-d** shows no dyes could be detected at 50 mM borate concentration except alizarin, which eluted at 25 minute with a very small fluorescence signal (less than 0.1 a.u.).

Therefore, by increasing the concentration the run time had increased and the fluorescence properties of most dyes had decreased. Generally speaking, by increasing the ionic strength the conductivity of the solution increases which in return causes Joule heating. Joule heating causes an increase in temperature, which results in decreasing the viscosity. Therefore, ionic strength has an influence on EOF and mobility of the eluent. The increase in migration time can be explained by decreasing the mobility of the eluents. The charged eluents are surrounded by the counterions of solution and the mobility direction of counterions and eluents are different. By increasing the ionic strength of the solution, the numbers of counterions are increased and they drag the charged eluents toward the opposite direction, which causes the longer migration time [22]. The best baseline separation within reasonable migration time was achieved using 20 mM borate buffer. For this research, the concentration of buffer was defined as 20 mM.

2.4.4 Separation voltage

The first approach was to study the effect of voltage on separation of selected dyes. In the preliminary study a mixture of 3 dyes: alizarin $5 \mu\text{g ml}^{-1}$, carmine $10 \mu\text{g ml}^{-1}$ and morin $0.5 \mu\text{g ml}^{-1}$ were prepared in borate buffer at 20 mM (pH = 9.2) and 3 voltages were investigated: 16, 18 and 20 kV. The reason for choosing these voltages is as follows: in study of

buffer concentration by applying 16 kV the separation was achieved in 25 minutes which could be considered as a long practical time in this pilot study. Therefore, 16 kV was chosen as minimum voltage to avoid longer migration time in lower voltages. In order to define the maximum voltage Ohm's law is used. The changes of current were plotted as a function of voltage and when deviation from linearity happened (at 21 kV), it meant the voltage reached to the level, which generates Joule heating (**Figure 2.6**). Moreover, the separation of dyes above 20 kV in summer time was not always successful because of the increment of noise due to the interaction of moisture in the air with the electric field along the capillary.

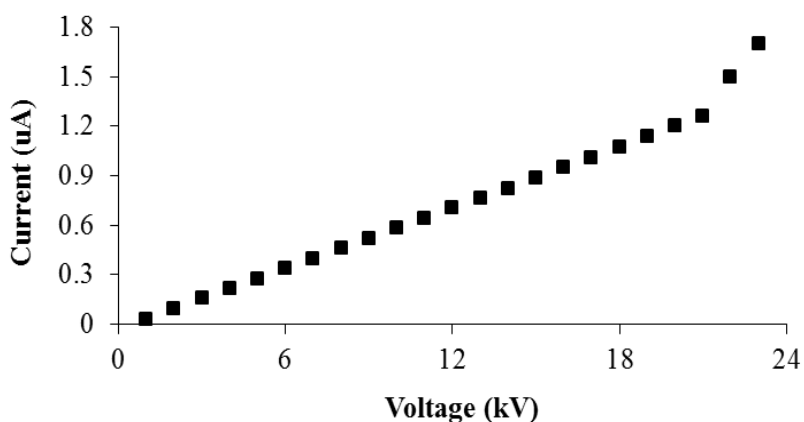


Figure 2.6 Ohm's law plot for 20 mM borate buffer with 20 mM SDS at pH= 9.2

As it can be seen in **Figure 2.7** by increasing voltage with 2 kV increments, the run time decreased about 2 minutes; therefore, by using 20 kV the separation was achieved in shorter time. Therefore, to study the effect of buffer pH and modifier, the separation was studied using 16 kV.

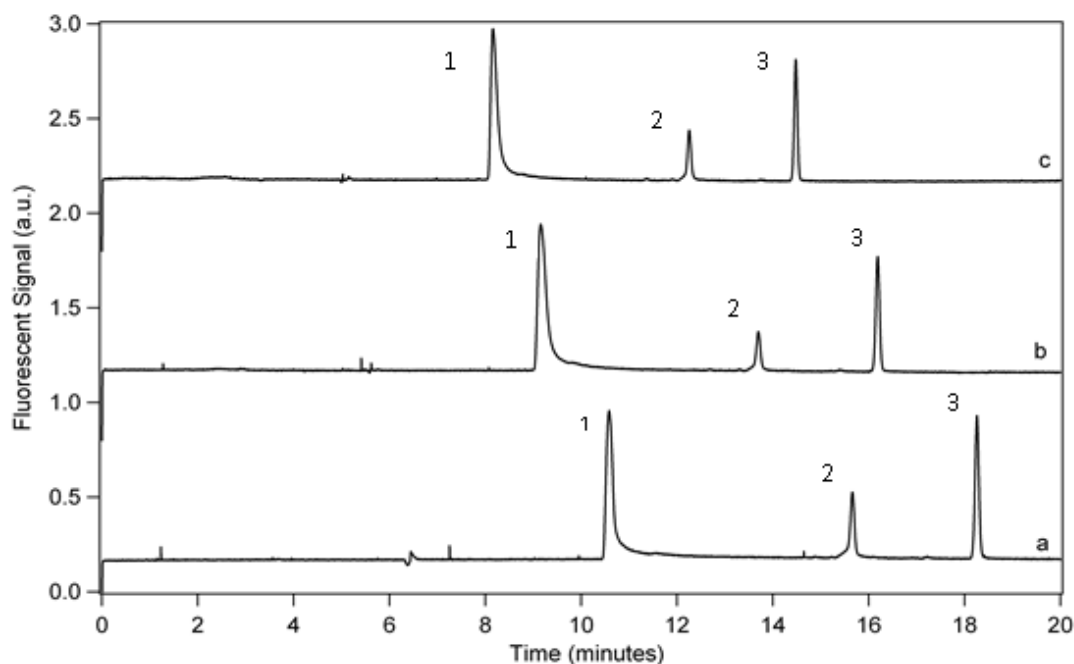


Figure 2.7 Separation of (1) $5 \mu\text{g ml}^{-1}$ alizarin, (2) $10 \mu\text{g ml}^{-1}$ carmine and (3) $0.5 \mu\text{g ml}^{-1}$ morin using 3 applied voltages **(a)** 16 kV **(b)** 18 kV and **(c)** 20 kV, using borate buffer 20 mM with 20 mM SDS (pH = 9.2).

2.4.5 pH studies

In the next part, the effect of different pH of 20 mM borate buffer with 20 mM SDS was investigated. The applied voltage for separation was 16 kV. **Figure 2.8** shows the separation of 4 dyes at pH 8.0, 8.5, 9.2 and 10.0. According to **Figure 2.8-a** at pH= 8.0 the peak for alizarin is very small and could not be detected since it had overlapped with the beginning of the tailing peak of purpurin, also carmine overlapped with the end of the tailing peak of purpurin.

By increasing the pH to 8.5, the elution time increased. At this pH alizarin could be differentiated from purpurin, since purpurin showed a sharper peak but the baseline separation had not been achieved. Regarding carmine and morin, they showed better baseline separation compared to pH= 8.0. **Figure 2.8-c** shows the separation of dyes at pH= 9.2. As

can be seen, the baseline separation of dyes was achieved, but still the peak shape of purpurin was not desirable since it had spread out.

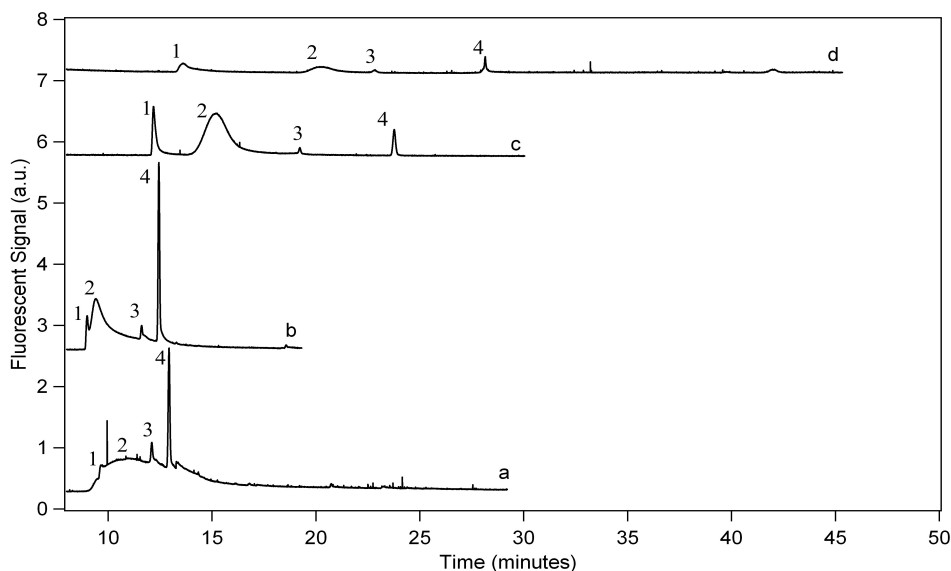


Figure 2.8 Separation of (1) $5 \mu\text{g ml}^{-1}$ alizarin, (2) $5 \mu\text{g ml}^{-1}$ purpurin, (3) $10 \mu\text{g ml}^{-1}$ carmine and (4) $5 \mu\text{g ml}^{-1}$ morin at 16 kV, using different borate buffer pH values with 20 mM SDS: (a) pH= 8.0, (b) pH= 8.5, (c) pH= 9.2 and (d) pH= 10.0.

By increasing the pH to 10.0, the fluorescence properties of dyes decreased dramatically. This may have been due to the photochemical instability of dyes at this pH, which resulted in breaking down the molecule of dyes, decreasing their concentrations and in return decreasing their fluorescence.

The pH studies showed that the migration time and pH were related and were consistent with the results from another research group [12]. It was proven that at higher pH, Joule heating occurs and that the negative charges on dyes increase. It is the reason for greater affinity of negatively charged eluents for the borate buffer, which resulted in formation of a complex between phenolic group in dyes with borate buffer [23].

Moreover, the separation of dyes at different pH values 8.0, 9.2 and 10.0 using higher voltage 20 kV was investigated. The results from this study are presented in **Figure 2.9**,

which shows by increasing the voltage the run time effectively decreased. For instance, in case of pH= 8.0 and 9.2 using 20 kV the dyes eluted 5 minutes earlier than using 16 kV. Moreover, using 20 kV applied voltage improved the peak shapes of all dyes except for carmine. It should be mentioned all dyes showed sharper peaks in both pH= 8.0 and 9.2 buffer. In spite of improving the peak shapes using pH= 8.0, there was no baseline separation and the dyes eluted all together.

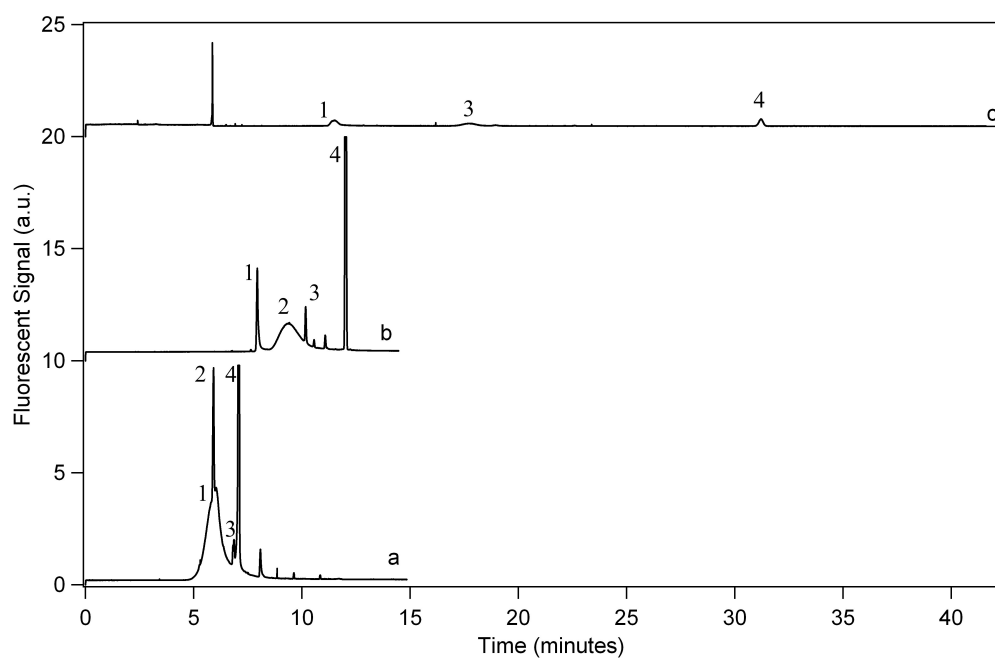


Figure 2.9 Separation of (1) $5 \mu\text{g ml}^{-1}$ alizarin, (2) $5 \mu\text{g ml}^{-1}$ purpurin, (3) $10 \mu\text{g ml}^{-1}$ carmine and (4) $5 \mu\text{g ml}^{-1}$ morin at 20 kV, using different borate buffer pH values with 20 mM SDS :**(a)** pH= 8.0, **(b)** pH= 9.2, **(c)** pH= 10.0.

Increasing the voltage at pH= 10.0 did not improve the results and it appears that the dyes had lost their fluorescence properties at this pH. This observation as explained previously could be due to photochemical instability of dyes at this pH. Similar to using pH= 10.0 with 16 kV, the results from using pH= 10.0 with 20 kV were inconsistent and increasing the voltage did not improve the fluorescence properties of the dyes.

According to the results from pH studies, it was decided to investigate the effect of modifier at different concentrations on separation at pH= 8.5.

2.4.6 The Effect of modifier

Low concentrations of organic solvents (as %) can have profound effects on the colligative properties of running buffers used in CE. Organic solvents affect the conductivity and viscosity of buffer solutions in the capillary and therefore, have a dramatic effect on the separation of target compounds. For example, organic solvents such as methanol or acetonitrile have been used as chemical modifiers for improving peak shape and improving peak resolution, primarily as a result of longer migration times [24]. Other researchers have reported that improved separations of anthraquinone dyes can be achieved with 10% acetonitrile in 30 mM sodium borate [3].

In a preliminary study the changes of fluorescence of alizarin, purpurin, carmine and morin as a function of percentage of different modifiers including methanol, acetonitrile and isopropanol were studied. The results of these studies are given in **Figure 2.10**.

As shown in **Figure 2.10** there was a relationship between fluorescence properties and the percentage of modifiers. The fluorescence intensities did generally decrease as the amount of modifier increased, this was true for the fluorescence of most dyes with the exception of purpurin in the presence of acetonitrile. It was concluded that by increasing the amount of methanol and isopropanol the fluorescence decreased at the same average percentage points, which were -0.14, -0.067, - 0.045 respectively for alizarin, purpurin and carmine. From this data it was concluded that alizarin was suppressed most and carmine least.

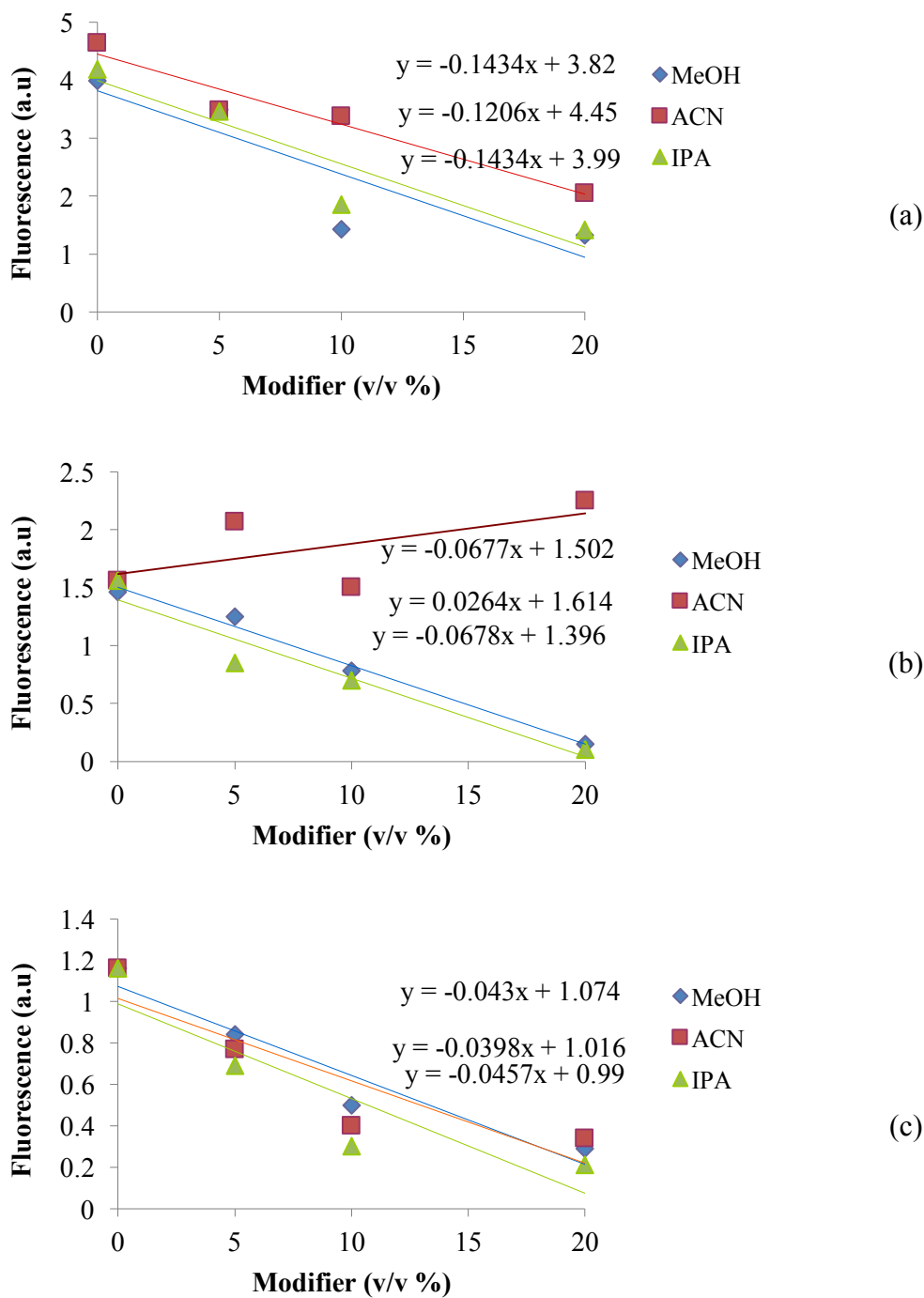


Figure 2.10 a, b, c The fluorescence changes of dyes as a function of modifier percentages: methanol (MeOH), acetonitrile (ACN) and isopropanol (IPA), **(a)** 5 $\mu\text{g ml}^{-1}$ alizarin, **(b)** 10 $\mu\text{g ml}^{-1}$ purpurin, **(c)** 5 $\mu\text{g ml}^{-1}$ carmine, at 16 kV, using 20 mM borate buffer concentration at pH= 9.2 with 20 mM SDS.

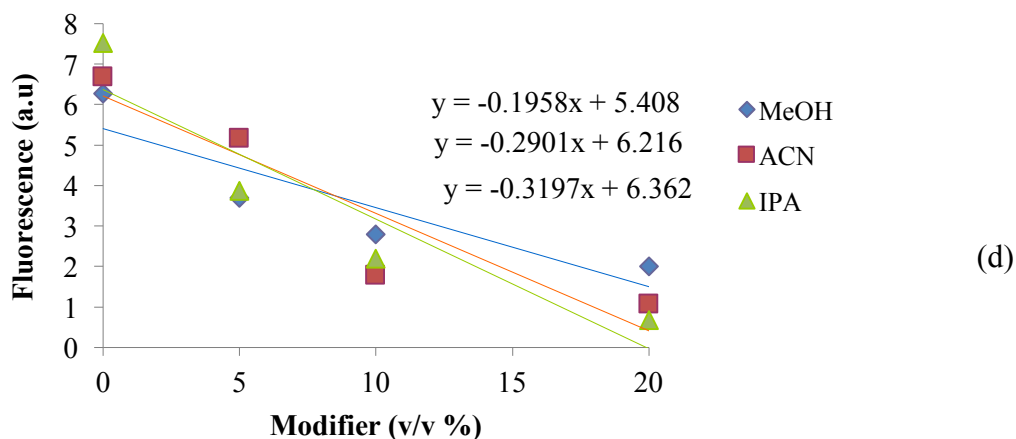


Figure 2.10 d The fluorescence changes of $2 \mu\text{g ml}^{-1}$ morin as a function of modifier percentages: methanol (MeOH), acetonitrile (ACN) and isopropanol (IPA) at 16 kV, using 20 mM borate buffer concentration at pH= 9.2 with 20 mM SDS.

These graphs indicated that the effect of acetonitrile in decreasing the fluorescence intensity of alizarin and carmine was less than the other 2 modifiers. The rate of fluorescence changes for carmine was less than alizarin (-0.039 vs. -0.12). However, the fluorescence changes of purpurin did not show the same trend and in the presence of 20% acetonitrile it fluoresced more intensely. This result was in agreement with the results reported by Miliani *et al.* [25]. Regarding the changes of fluorescence intensity of morin as a function of percentage of modifiers, it was concluded that the fluorescence decreased as follows: isopropanol > acetonitrile > methanol. (**Figure 2.10-d**).

Moreover, there was an inverse variation between the changes of current as a function of modifier percentage. By comparing the slopes of **Figure 2.11** it was concluded that the current was decreased more by isopropanol than by methanol or acetonitrile.

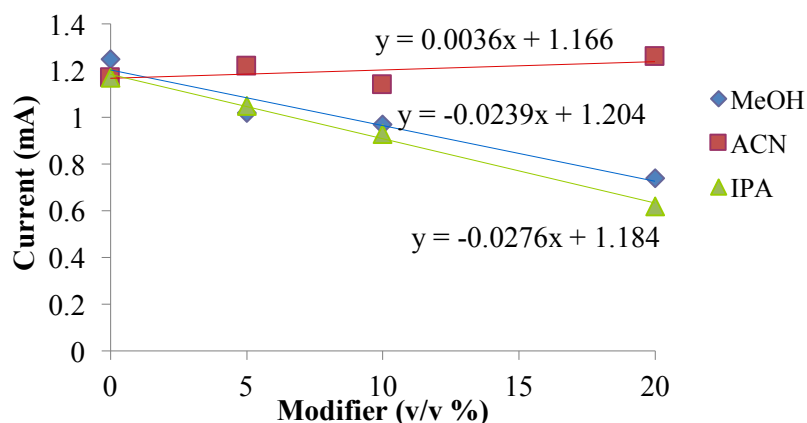


Figure 2.11 The changes of current as a function of modifier percentage methanol (MeOH), acetonitrile (ACN) and isopropanol (IPA) at 16 kV, using 20 mM borate buffer concentration at pH= 9.2 with 20 mM SDS.

Also, the changes of migration time of dyes as the function of modifier percentage were studied. The results of these studies were given in **Figures 2.12 (a-d)**. This study showed that all dyes had a longer migration time in the presence of isopropanol, and methanol than acetonitrile. The data showed that morin had an increased migration time compared to other dyes.

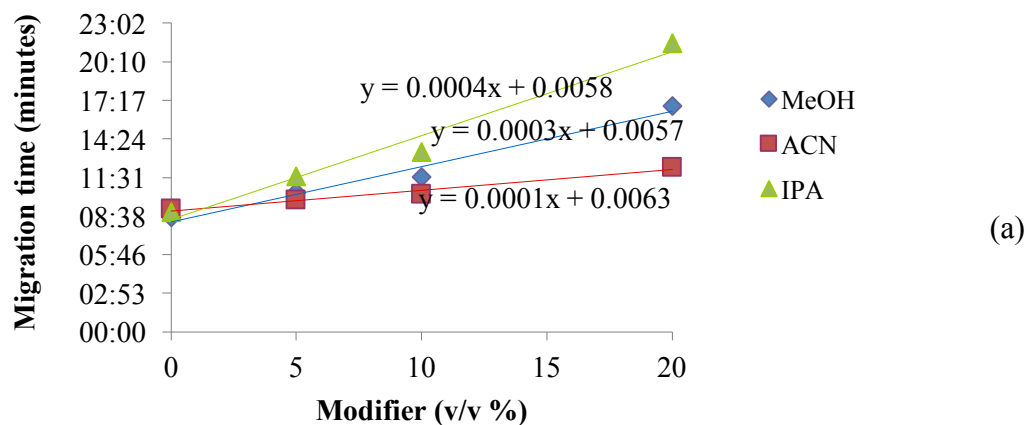


Figure 2.12 a Migration time changes of 5 $\mu\text{g ml}^{-1}$ alizarin as a function of modifier percentages: methanol (MeOH), acetonitrile (ACN) and isopropanol (IPA), at 16 kV, using 20 mM borate buffer concentration at pH= 9.2 with 20 mM SDS.

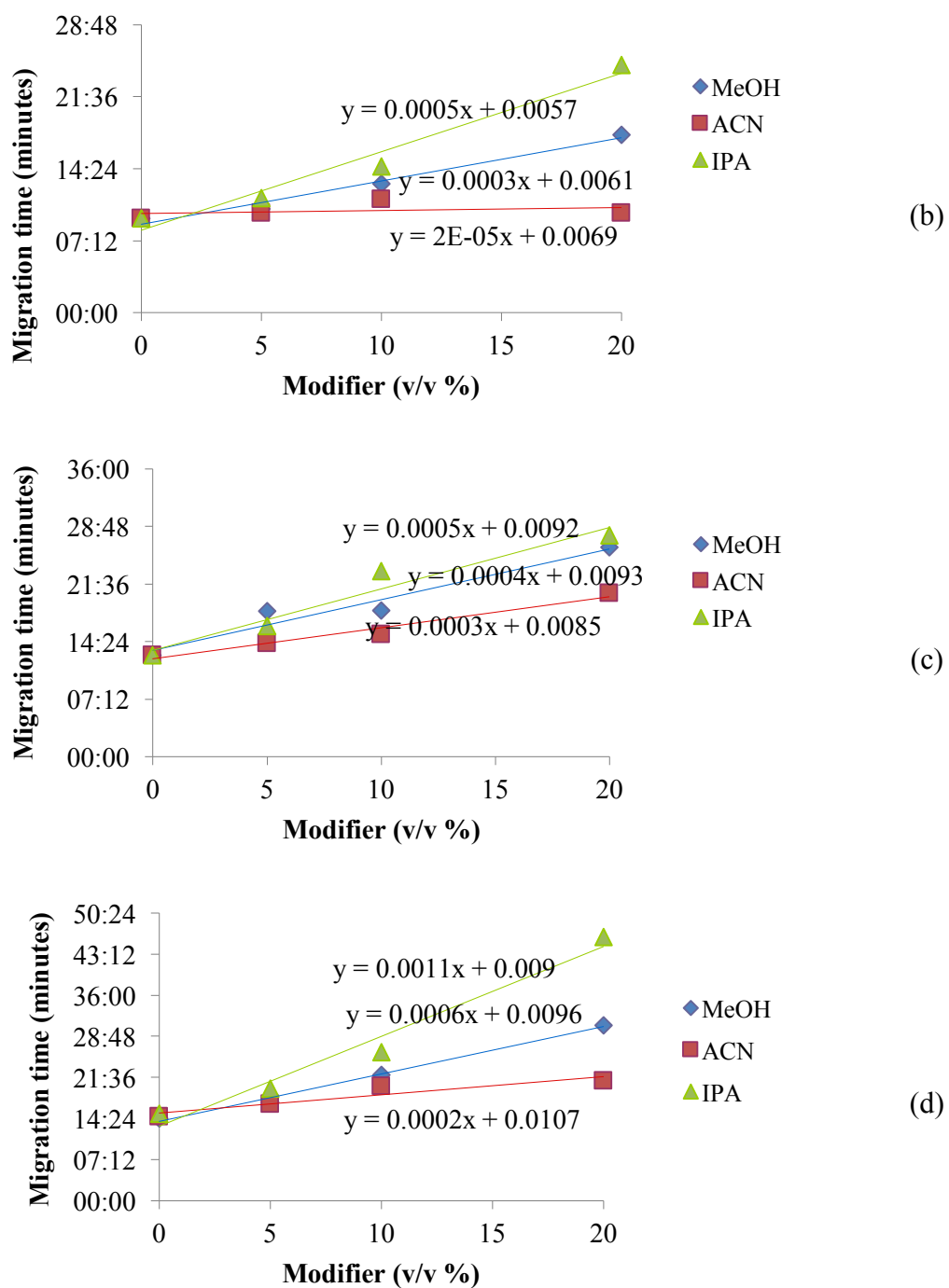


Figure 2.12 b, c, d Migration time changes of dyes as a function of modifier percentages: methanol (MeOH), acetonitrile (ACN) and isopropanol (IPA), **(b)** $10 \mu\text{g ml}^{-1}$ purpurin, **(c)** $5 \mu\text{g ml}^{-1}$ carmine, **(d)** $2 \mu\text{g ml}^{-1}$ morin at 16 kV, using 20 mM borate buffer concentration at pH= 9.2 with 20 mM SDS.

In conclusion, the presence of isopropanol as a modifier caused the greatest decrease in current and consequently increased the migration time. Therefore, in this study isopropanol was not considered as a viable modifier.

According to results from pH studies (**Figure.2.8**), it was concluded that at pH= 8.5 the individual dyes could be detected; alizarin and carmine showed overlapping respectively at the beginning and end of purpurin's tailing peak. Therefore, it was decided to study whether adding modifier could improve the separation of these dyes at pH= 8.5.

In one study the effect of 2 different common modifiers was investigated: methanol and acetonitrile. The results from this study are presented in **Figure 2.13**. It shows that the application of methanol for separation of 4 dyes was achieved in a longer run time in comparison to application of acetonitrile. Additionally, using methanol suppressed fluorescence signals of dyes more than acetonitrile.

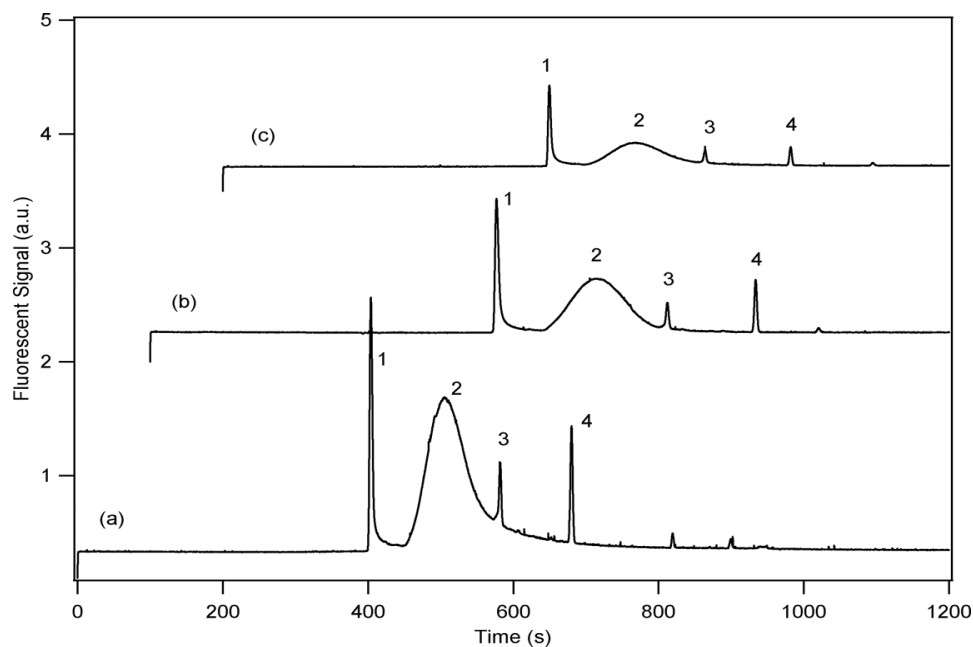


Figure 2.13 Separation of (1) $5 \mu\text{g ml}^{-1}$ alizarin, (2) $10 \mu\text{g ml}^{-1}$ purpurin, (3) $10 \mu\text{g ml}^{-1}$ carmine and (4) $0.5 \mu\text{g ml}^{-1}$ morin in (a) 20 mM borate buffer, (b) 20 mM borate and 5% acetonitrile and (c) 20 mM borate and 5% methanol. Adapted from Ref. [20] – Copyright: Taylor and Francis.

The effect of adding acetonitrile at different concentrations 5, 10, and 20% (v/v) to the mixture of dyes using 20 mM borate buffer at pH= 8.5 (**Figure. 2.14**) with 20 mM SDS was investigated.

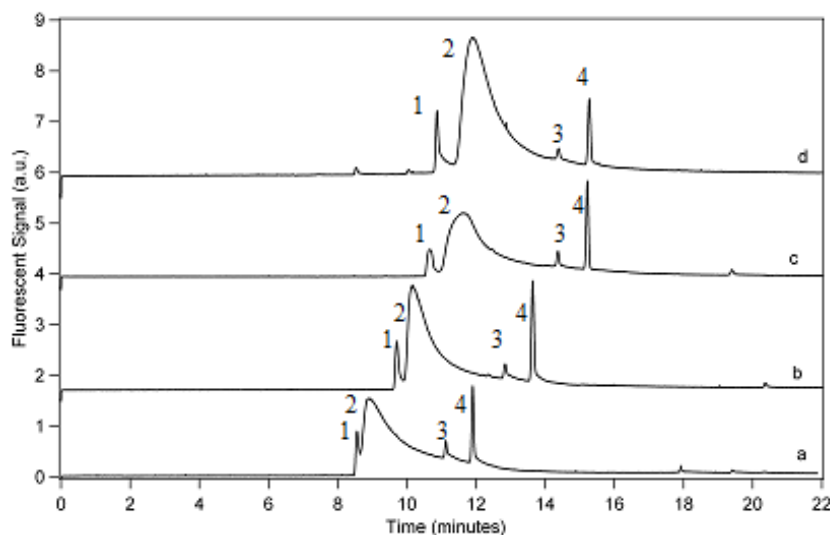


Figure 2.14 Separation of (1) $5 \mu\text{g ml}^{-1}$ alizarin, (2) $5 \mu\text{g ml}^{-1}$ purpurin, (3) $10 \mu\text{g ml}^{-1}$ carmine and (4) $1 \mu\text{g ml}^{-1}$ morin at 16 kV, using borate buffer pH= 8.5 with 20 mM SDS, with different concentration of acetonitrile as modifier (a) no modifier, (b) 5% (c) 10% and (d) 20%.

According to **Figure 2.14** at pH= 8.5, in spite of increasing the elution time by increasing the amount of modifier, the best baseline separation was achieved at 20% acetonitrile. The peak size of purpurin showed enlargement.

Since the presence of 20% acetonitrile at pH= 8.5 with separation voltage of 16 kV resulted in baseline separation of 4 dyes, the same condition of separation was investigated for pH 7.0, 8.0, 8.5 and 9.2. The results of these separations are summarized in **Figure 2.15**. As it can be seen in **Figure 2.15-a**, the presence of 20% acetonitrile did not improve the resolution of the peaks at pH= 7.0. Without the presence of modifier, best baseline

separation of dyes was achieved at pH= 8.5. However, it was observed that the best separation would result at pH= 8.0 by adding 20% acetonitrile. The resulting elution time was shorter and the peak shape of purpurin became sharper compared to its shape at pH= 8.5.

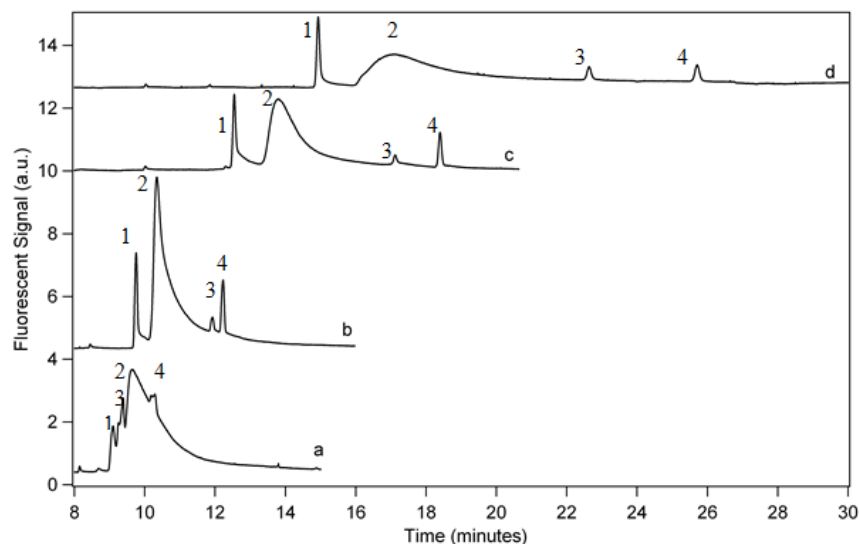


Figure 2.15 Separation of (1) $5 \mu\text{g ml}^{-1}$ alizarin, (2) $5 \mu\text{g ml}^{-1}$ purpurin, (3) $10 \mu\text{g ml}^{-1}$ carmine and (4) $1 \mu\text{g ml}^{-1}$ morin at 16 kV, using 20 % acetonitrile at different borate buffer pH values with 20 mM SDS: (a) pH= 7.0, (b) pH= 8.0, (c) pH= 8.5 and (d) pH= 9.2.

Figure 2.15-d depicted that the presence of 20% acetonitrile at pH= 9.2 increased the migration time, which could improve the separation of dyes.

2.4.7 Voltage revisited

Applied voltage is another parameter, which has a great effect on CE separation.

As *Eq. 1.6* and *Eq. 1.13* show the electroosmotic and electrophoretic mobility are related to voltage, and by increasing square feet of voltage *Eq. 1.17*, while maximum voltage should be in the criteria to avoid Joule heating.

Since the results of adding 20% acetonitrile to different pH values using 16 kV showed that the best peak shapes of dyes were achieved at pH= 8.0, it was decided to study the

effect of voltage on separation of dyes. The voltages for this study were 14, 18 and 20 kV. These voltages were chosen by considering the results from preliminary study of voltage at pH= 9.2 (**Figure 2.7**). Since at pH= 8.0 the dyes eluted faster than at pH= 9.2, it was decided to check voltage 14 kV. Also, by plotting Ohm's law diagram (**Figure 2.16**), it was concluded that the maximum voltage of 20 kV could be used.

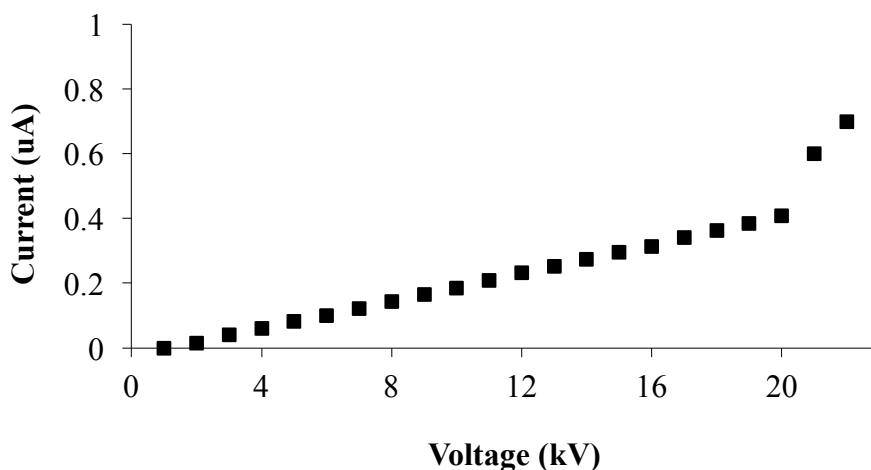


Figure 2.16 Ohm's law plot for 20 mM borate buffer with 20 mM SDS at pH= 8.0

As **Figure 2.17** depicts, by increasing the voltage, the run time has decreased: for example the first peak which belonged to alizarin eluted at minute 11:30 using 14 kV and by increasing 2 increments to 20 kV, alizarin eluted at minutes: 10:20, 8:50 and 8:10. Applying 20 kV (**Figure 2.17- d**) resulted in the best baseline separation as well as the sharpest and best peak shapes for all dyes. By applying higher voltage, the eluents migrate faster, and this is likely a reason that their peaks were sharper while purpurin was less spread out.

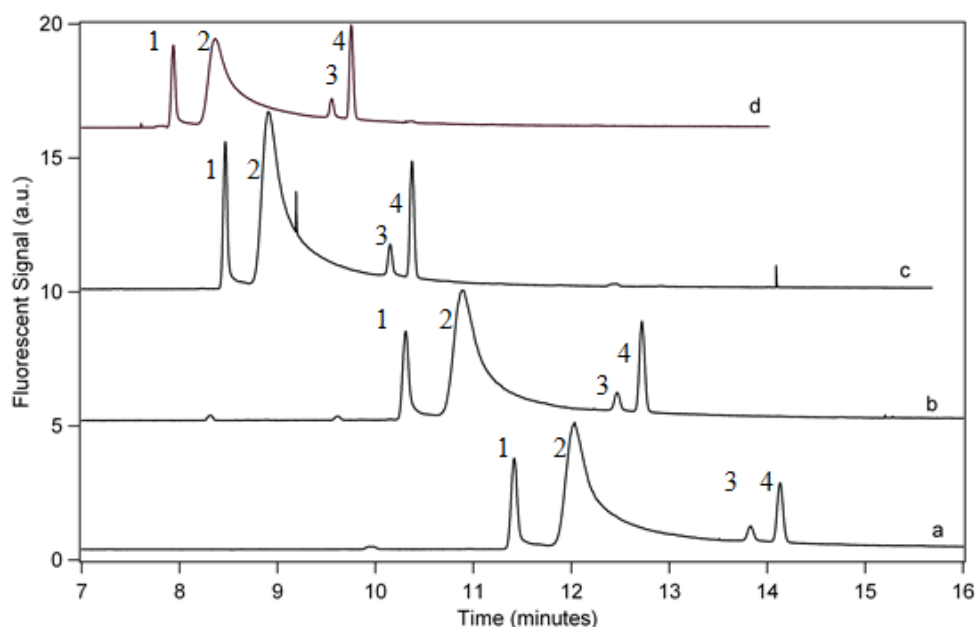


Figure 2.17 Separation of (1) $5 \mu\text{g ml}^{-1}$ alizarin, (2) $5 \mu\text{g ml}^{-1}$ purpurin, (3) $10 \mu\text{g ml}^{-1}$ carmine and (4) $1 \mu\text{g ml}^{-1}$ morin, using 20 % acetonitrile in borate buffer at pH= 8.0 with 20 mM SDS, using different applied voltages: **(a)** 14 kV, **(b)** 16 kV, **(c)** 18 kV and **(d)** 20 kV.

2.5 Conclusion

The objective of this chapter was to find out if CE-LIF could be applied for separation and identification of a selection of limited historically important dyes. In this study a collection of natural dyes which have been used in historical objects (textiles) was selected. These dyes were: 4 red anthraquinone dyes: alizarin, purpurin, carmine, and emodin, 8 yellow flavonoids; apigenin, luteolin, ap-7-*O*-glu, lu-7-*O*-glu (flavones), kaempferol, quercetin, morin, myricetin (flavonols), 1 yellow carotenoids dyes: crocin, 1 brown quinodic dye: lawson and 1 blue indigoids dye: indigo.

In CE-LIF the identification of dyes is based on their fluorescence properties which is affected by the excitation beam of laser source equipped with CE. Therefore, in order to choose the suitable available laser source which could be used for the dyes under this study,

the fluorescence property of each dye was examined using a fluorimeter. The selection of dyes was narrowed to anthraquinones, flavonoids, i.e. the dyes which did fluoresce using available laser sources. For preliminary studies the most important red and yellow dyes from anthraquinones and flavonoids were chosen: alizarin and purpurin the colourants in madder root, carmine the colourant in cochineal, and yellow morin dye belonging to flavonoids found in old fustic.

Since the available laser sources were at 488 nm, and 407 nm, therefore, the emission spectrum of each dye was collected using these 2 wavelengths. It was concluded the maximum emission wavelength of purpurin, carmine and morin was affected by the excitation wavelength and they showed red shifts when they were excited at 488 nm except for alizarin, where the excitation wavelength did not significantly affect the maximum emission wavelength. The results from this pilot study showed that 407 nm diode laser source was a preferred for most dyes.

Fluorometry studies also showed that the fluorescence intensity of alizarin and morin were more intense than other dyes and these results were in agreement with their electropherograms.

In order to remove scattered light and improve the detection limits of dyes different interference filters (500, 510, 535, 580, 615 and 630 nm) were installed in front of PMT. The 535 nm filter was found the best filter for all dyes which provided the greatest amount of signal to noise ratio for most dyes.

As mentioned before, separation in CE is affected by: the pH and concentration of buffer, the concentration of micelles, type and concentration of modifier, and applied voltage. According to literature [11, 12, 14, 26] CE in mode of MEKC with borate buffer at

pH= 9.2 and SDS was favorable for studying anthraquinone dyes. Therefore, borate buffer with 20 mM SDS was chosen as back ground electrolyte (BGE). The concentration of borate buffer was manipulated to achieve the baseline separation of dyes (applied voltage= 16 kV). Increasing the concentration of borate buffer from 10 mM to 50 mM resulted in longer migration time with inferior fluorescence intensity of dyes. The baseline separation of the 4 dyes at shortest time with reasonable fluorescence intensity was achieved using 20 mM borate buffer.

Another experimental parameter in CE separation is applied voltage. Three voltages were investigated: 16, 18 and 20 kV. It was observed when 16 kV was applied with borate buffer at pH=9.2 and the separation was achieved in 25 minutes, therefore 16 kV was chosen as minimum voltage to avoid longer migration time. The maximum voltage was defined using Ohm's law and plotting the current changes as the function of voltage, when deviation from linearity happened (at 21 kV), it meant the voltage reached to the level, which generates Joule heating. Baseline separation was achieved at 20 kV in shorter time.

The role of buffer pH was also studied when the applied voltage was 16 k. The separation of 4 dyes was investigated using pH values: 8.0, 8.5, 9.2 and 10.0. This study showed that the migration time and pH were related proportionally and were consistent. By increasing the pH= 10.0 the fluorescence intensity of dyes decreased dramatically, which might have been due to photochemical instability of dyes at this pH. It was observed that at lower pH the baseline separation was not achieved and dyes co-eluted. The same separation study was performed using 20 kV applied voltage, where resulted in shorter migration time and since dyes moved faster, the peak shapes of all dyes at pH< 9.2 were improved except for

carmine. Therefore, the effect of modifier at different concentrations on separation of alizarin, carmine and morin at pH= 8.5 was investigated. It is worthy noted that low concentrations of organic solvents (as %) can have profound effects on the colligative properties of running buffers used in CE. In this preliminary study the changes of fluorescence intensity of alizarin, purpurin, carmine and morin as a function of percentage of different modifiers including methanol, acetonitrile and isopropanol were studied. The fluorescence intensities did generally decrease as the amount of modifier increased, this was true for the fluorescence of most dyes except for purpurin in the presence of acetonitrile. It was concluded that the trend of decreasing the fluorescence intensity of dyes was: isopropanol > methanol > acetonitrile. Furthermore, the effect of modifier percentage on the current was studied and it showed that the current was decreased more by isopropanol than by methanol or acetonitrile. The effect of modifier on the migration time of the dyes was also explored and it was concluded that all dyes had a longer migration time in the presence of isopropanol, and methanol than acetonitrile. Since, the presence of isopropanol as a modifier caused the greatest decrease in current and consequently increased the migration time. Therefore, isopropanol was not considered as a viable modifier. The effect of 5% methanol and 5% acetonitrile were studied on the separation of 4 dyes at pH= 8.5, applied voltage of 16 kV. The results showed that the application of methanol for separation of 4 dyes was achieved in a longer run time in comparison to application of acetonitrile. Additionally, using methanol suppressed fluorescence signals of dyes more than acetonitrile. The effect of higher concentration of acetonitrile was also investigated on the separation of 4 dyes using the same separation conditions (pH= 8.5, V= 16 kV). In spite of increasing the elution time by

increasing the amount of modifier, the best baseline separation was achieved at 20% acetonitrile. The same experiment was performed on the mixture of dyes at pH values: 7.0, 8.0, and 9.2. The presence of 20% acetonitrile did not improve the resolution of the peaks at pH= 7.0. It was also observed that the best separation would result at pH= 8.0 by adding 20% acetonitrile. The resulting elution time was shorter and the peak shape of purpurin became sharper compared to its shape at pH= 8.5.

Therefore, the optimum condition for baseline separation of the 4 dyes under this study was defined by manipulating all these factors. The results from this study would be used for separation a selection of more than 4 dyes.

Capillary electrophoresis is an effective tool for the separation and detection of small quantities of dye mixtures. From this work, it was observed that baseline separation of natural dyes could be achieved using CE in MEKC mode. This was also true for dyes that are very similar structurally such as alizarin and purpurin. When compared with to other dyes, the behavior of purpurin was unusual. The peaks for purpurin were exceptionally broad with elution times of ~1 minute. Attempts to improve the peak shape with chemical modifiers failed. The unusual peak shape of purpurin indicates that chemically, it behaves quite differently from alizarin at high pH. A second unusual property of purpurin is related to the reproducibility of peak heights. Over time, the purpurin peak height decreases significantly which strongly suggests that degradation may be occurring, particularly at high pH.

As a result of this preliminary study, chemical properties such as pH and the concentration of organic solvents have a profound effect on the retention time and fluorescence of

target compounds such as alizarin or purpurin. Therefore, further study on separation of mixtures of different dyes is pursued in Chapter 3.

2.6 References

1. Song-gang, J., et al., *Separation and determination of anthraquinone derivatives in rhubarb and its preparations by micellar electrokinetic capillary chromatography*. Biomedical Chromatography, 1998. **12**(6): p. 335-337.
2. Watanabe, T. and S. Terabe, *Analysis of natural food pigments by capillary electrophoresis*. Journal of Chromatography A, 2000. **880**(1): p. 311-322.
3. Weng, W.C. and S.J. Sheu, *Separation of Anthraquinones by Capillary Electrophoresis and High-Performance Liquid Chromatography*. Journal of High Resolution Chromatography, 2000. **23**(2): p. 143-148.
4. Shang, X. and Z. Yuan, *Determination of active components in rhubarb and study of their hydrophobicity by micellar electrokinetic chromatography*. Bioorganic & medicinal chemistry letters, 2003. **13**(4): p. 617-622.
5. Li, F., Q.-E. Cao, and Z. Ding, *Separation and determination of five anthraquinones in rheum and rheum-containing preparations by capillary zone electrophoresis*. Chromatographia, 2004. **59**(11-12): p. 753-757.
6. Jiang, T.F., Z.H. Lv, and Y.H. Wang, *Separation and determination of anthraquinones in Cassia obtusifolia (Leguminosae) by micellar electrokinetic capillary electrophoresis*. Journal of separation science, 2005. **28**(16): p. 2225-2229.
7. Koyama, J., et al., *Simultaneous Determination of Anthraquinones, Their 8-BETA.-D-Glucosides, and Sennosides of Rhei Rhizoma by Capillary Electrophoresis*. Chemical and pharmaceutical bulletin, 2005. **53**(5): p. 573-575.
8. Sun, S.-W. and P.-C. Yeh, *Analysis of rhubarb anthraquinones and bianthrone by microemulsion electrokinetic chromatography*. Journal of pharmaceutical and biomedical analysis, 2005. **36**(5): p. 995-1001.
9. Tian, K., et al., *Determination of five anthraquinones in medicinal plants by capillary zone electrophoresis with beta-cyclodextrin addition*. J Chromatogr A, 2006. **1123**(1): p. 134-7.
10. Maguregui, M., et al., *Micellar electrokinetic chromatography method for the determination of several natural red dyestuff and lake pigments used in art work*. Journal of Chromatography A, 2007. **1154**(1): p. 429-436.
11. Svobodová, E., et al., *Use of capillary zone electrophoresis and micellar electrokinetic chromatography for separations of anthraquinone derivatives*. Analytical Letters, 2011. **44**(10): p. 1783-1795.
12. Trojanowicz, M., L. Wójcik, and K. Urbaniak-Walczak, *Identification of natural dyes in historical Coptic textiles by capillary electrophoresis with diode array detection*. Chemia analityczna, 2003. **48**(3): p. 607-620.

13. López-Montes, A., et al., *Simultaneous identification of natural dyes in the collection of drawings and maps from The Royal Chancellery Archives in Granada (Spain) by CE*. Electrophoresis, 2007. **28**(8): p. 1243-1251.
14. López-Montes, A., et al., *Characterization of sepia ink in ancient graphic documents by capillary electrophoresis*. Microchemical Journal, 2009. **93**(2): p. 121-126.
15. Puchalska, M., et al., *Identification of anthraquinone coloring matters in natural red dyes by electrospray mass spectrometry coupled to capillary electrophoresis*. Journal of mass spectrometry, 2003. **38**(12): p. 1252-1258.
16. Surowiec, I., W. Nowik, and M. Trojanowicz, *Post-column deprotonation and complexation in HPLC as a tool for identification and structure elucidation of compounds from natural dyes of historical importance*. Microchimica Acta, 2008. **162**(3-4): p. 393-404.
17. Van Hecke, G.R. and K.K. Karukstis, *A guide to lasers in chemistry*. 1998: Jones and Bartlett.
18. Camilleri, P., *Capillary electrophoresis: theory and practice*. 1997: CRC press.
19. Arriaga, E.A., Y. Zhang, and N.J. Dovichi, *Use of 3-(p-carboxybenzoyl) quinoline-2-carboxaldehyde to label amino acids for high-sensitivity fluorescence detection in capillary electrophoresis*. Analytica chimica acta, 1995. **299**(3): p. 319-326.
20. Goltz, D.M., et al., *Separation of historical dyes using capillary electrophoresis with laser-induced fluorescence detection*. Journal of Liquid Chromatography & Related Technologies, 2012. **35**(14): p. 2054-2065.
21. Gupta, M.N., et al., *Polarity Index: The Guiding Solvent Parameter for Enzyme Stability in Aqueous-Organic Cosolvent Mixtures*. Biotechnology progress, 1997. **13**(3): p. 284-288.
22. Weinberger, R., *Practical capillary electrophoresis*. 2000: Academic Press.
23. Kapnissi-Christodoulou, C.P., *Method Development by Use of Capillary Electrophoresis and Applications in Pharmaceutical, Biological and Natural Samples*. 2012: INTECH Open Access Publisher.
24. JoonáLee, Y., *Effect of organic solvents on the separation of benzoic acids by capillary electrophoresis*. Analyst, 1995. **120**(11): p. 2689-2694.
25. Miliani, C., A. Romani, and G. Favaro, *Acidichromic effects in 1, 2-di-and 1, 2, 4-trihydroxyanthraquinones. A spectrophotometric and fluorimetric study*. Journal of Physical Organic Chemistry, 2000. **13**(3): p. 141-150.
26. Zlotnick, J. and F. Smith, *Separation of some black rollerball pen inks by capillary electrophoresis: preliminary data*. Forensic Science International, 1998. **92**(2): p. 269-280.

Chapter 3

The application of MEKC-LIF for separation of selected red and yellow historical dyes

Micellar Electrokinetic Capillary Chromatography with Laser-Induced Fluorescence Detection for Separation of Red and Yellow Historical Dyes

Shokoufeh Ahmadi^{1,2}, Douglas B. Craig² and Douglas M. Goltz^{1,2}

1. Department of Chemistry, University of Manitoba, Winnipeg, MB, R3T 2N2 CANADA
2. Department of Chemistry, Richardson College for the Environment and Science Complex, University of Winnipeg, 599 Portage Ave., Winnipeg, MB, R3B 2G3 CANADA

Individual contribution of authors:

Shokoufeh Ahmadi performed all analysis/separations, data collection, data interpretation as well as drafted the manuscript for publication.

Dr. Doug Goltz supervised the development of experiments and helped edit the manuscript. He was the corresponding author.

Dr. Doug Craig made a contribution experimentally and helped edit the manuscript prior to publication.

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3.1 Introduction

In this chapter, the separation parameters in MEKC-LIF were optimized for the separation of red and yellow historical dyes in 20 mM borate buffer with 20 mM sodium dodecyl sulfate (SDS). Separation conditions were optimized by changing pH, organic modifier (methanol and acetonitrile) concentrations, and applied voltage. The mixtures of dyes used in this study included: 4 anthraquinone dyes (alizarin, purpurin, emodin, carmine) and 6 flavonoid dyes (luteolin, apigenin, ap-7-*O*-glu, lu-7-*O*-glu, kaempferol, quercetin, morin, and myricetin). For this work, dyes were introduced electro-kinetically (10 kV for 5 s) into a 50 cm capillary (10 μ m id) and separated using a range running of potentials: 18, 20, 22, 25 kV. Anthraquinone dyes, shown in **Figure 1.1** are typically red in appearance and are plant or animal based dyes [1]. Plant-based anthraquinones can be found in the madder root (*Rubia tinctorum*) and rhubarb (*Rheum rhabarbarum*). The dyes in madder consist primarily of alizarin and purpurin, while the dye in rhubarb consists of emodin. Cochineal and kermes dyes are examples of animal-based anthraquinones. Cochineal dyes are derived from the cochineal insect and consist of carminic acid. The lake form of carminic acid is commonly known as carmine and is often prepared using aluminum and calcium salts. Kermes is derived from the insect kermes vermilio and consists of kermesic acid, which is carminic acid aglycone[2].

Flavonoid dyes, shown in **Figure 1.2**, can be divided into 2 main groups: flavones and flavonols. Relative to anthraquinones, flavonoids are more prone to photo-chemically fading [3] and among the flavonoids, the flavonols tend to be less resistant to fading than flavones. Examples of flavone based yellow dyes are weld and dyer's broom, which are

rich sources of luteolin and apigenin respectively. Old fustic, Persian berries, and walnut are examples of sources for flavonol dyes. Old fustic consists of morin and kaempferol. Persian berries are a good source for quercetin and kaempferol, while walnut is a rich source of myricetin [2],[4].

A number of instrumental approaches have been used to identify and characterize dyes in historical objects. These include ultraviolet-visible (UV-Vis) reflectance spectroscopy [5-8], Fourier transform infrared spectroscopy [9-11] and Raman spectroscopy [12, 13]. The lack of specificity and poor sensitivity of these approaches make them less than ideal for detecting small quantities of dye. A number of separation techniques have also been explored and these include: thin layer chromatography (TLC) [14, 15] high performance liquid chromatography (HPLC) [16, 17], gas chromatography (GC) [18] and capillary electrophoresis (CE) [19-26]. HPLC is a common technique for separation of dyes and can be equipped with many different detection schemes including UV-Vis and fluorescence detection, photodiode array (DAD) and mass spectrometry (MS). In terms of application of GC for studying dyes, many dyes need to be derivatized as they are often large, polar molecules [27].

Regarding application of CE for separation of historical dyestuffs relatively few papers have been published [21, 28-31]. The separation of anthraquinones and flavonoids using CE with MS detection has been described by Surowiec [20]. Capillary electrophoresis using micelles or micellar electrokinetic chromatography (MEKC) has been described for the separation of anthraquinones and flavonoids [21]. Maguregui [31] reported the separation of red dyes using MEKC. The separation of mixtures of red, yellow and blue dyes using MEKC has also been described [32].

However, for the first time the feasibility study of application CE-LIF was explored in our lab to study selected historical dyes. From previous work (Chapter 2) it was concluded that CE-LIF could be used for separation and identification of 4 historically important dyes including alizarin, purpurin, carmine and morin. This pilot study showed that 407 nm laser source with 535 nm optical filter could be used for exciting the dyes. The background electrolyte was defined as 20 mM borate buffer with 20 mM SDS. The baseline separation of 3 dyes excluding purpurin, was achieved at pH values: 8.0, 8.5 and 9.2. However, when purpurin was in the mixture, it was an ongoing challenge to achieve the baseline separation between tailing peak of purpurin and carmine. Therefore, different parameters affecting CE separation were investigated, including: the pH of borate buffer, the acceptable range of applied voltage, the type and the range of concentration of modifiers. It was observed that the tailing peak of purpurin was improved by: lowering the pH (<9.2), and increasing the voltage ($V = 18$ kV). The baseline separation of purpurin and carmine was improved by adding 5% (v/v) acetonitrile. In this stage of the research the application of CE-LIF was investigated systemically on each class of dyes as well as a mixture of all of them. The objective of this phase of the research was to examine the feasibility of using CE-LIF for the separation and identification of the samples containing a more complicated mixture of dyes from different classes. Since, the desired colour in textiles was achieved by mixing different colourants, e.g. the green colour in textile was the result of mixing indigo and weld. Therefore, the separation of individual class of dyes as well as a mixture of them were studied at pH values: 8.0, 8.5 and 9.2. Separation of the mixture of different classes of dyes brought challenges due to the co-elution of at least 2 dyes with each other. Therefore, the effect of methanol and acetonitrile in different concentrations as modifiers, as well

as different applied voltages were investigated. A collection of natural dyes which have been used in historical objects (textiles) was selected as: 4 red anthraquinone dyes: alizarin, purpurin, carmine, and emodin, 8 yellow flavonoids; apigenin, ap-7-*O*-glu, lu-7-*O*-glu (flavones), kaempferol, quercetin, morin, myricetin (flavonols), 1 yellow carotenoids dyes: crocin, 1 brown quinodic dye: lawson and 1 blue indigoids dye: indigo. The fluorescence study of dyes excluded dyes based on their fluorescence properties at 407 nm, the wavelength of the available laser equipped with CE. The selection of dyes was narrowed to anthraquinones, flavonoids the dyes which did fluoresce using available laser sources. For preliminary studies in Chapter 2, the separation parameters were investigated on the most important red and yellow dyes from anthraquinones and flavonoids: alizarin and purpurin the colourants in madder root, carmine the colourant in cochineal, and yellow morin dye belonging to flavonoids found in old fustic.

3.2 Experimental

Separations were carried out using a laboratory-constructed CE instrument equipped with a post-column laser-induced fluorescence detection system [33]. The injection end of a 50 cm long, 10 μm inner diameter fused silica capillary (Polymicro Technologies) along with a Pt electrode connected to a high voltage supply (Spellman model CZE 2000), was placed into a buffer-filled vessel. The applied voltage across the capillary was typically 18, 20, 22, and 25 kV. The detection end of the capillary, from which a 1 mm length of the external polyimide coating was removed by flame, was inserted into a quartz sheath flow cuvette with a 250 by 250 μm inner bore (Hellma). The system was grounded through the sheath flow buffer within the cuvette. A diode laser (Coherent) with a 407 nm output (90 mW)

was employed. Light from the laser was focused with a 6.3 X, N.A. 0.2 microscope objective (Melles Griot) approximately 10 μm below the detection end of the capillary. Emission was collected at 90° using a 60 X, N.A. 0.7 microscope objective (Universe Kogaku), passed through a 535 AF45 optical filter (Omega Optical) and a slit and then onto a photomultiplier tube (PMT, Hamamatsu model 1477). The analog PMT signal was collected and digitized using a Pentium 4 computer through a PCI-MIO-16 XE I/O board utilizing LabVIEW software (National Instruments) at 10 Hz. The same board was used to control the electrophoresis voltage and PMT bias. Data was analyzed using IgorPro™ software.

3.3 Reagents

Dimethyl sulfoxide (DMSO), acetonitrile, sodium dodecyl sulfate (SDS) 99%, sodium hydroxide, were obtained from Sigma-Aldrich. Methanol was purchased from EMD Chemicals Inc. and sodium borate was obtained from Mallinckrodt. The dyes used in this study were purchased from different suppliers. Myricetin, apigenin, ap-7-*O*-glu, morin, purpurin and kaempferol were from Sigma-Aldrich, emodin was from Acros Organics and alizarin, carmine, quercetin, luteolin, lu-7-*O*-glu were from Alfa Aesar. Fluorescein (Molecular Probes) was diluted and used for aligning the optics of the CE-LIF. Borate buffers used in this study also contained 20 mM SDS in Milli-Q water (18 M Ω · cm). Borate buffers with varying pH were prepared using different ratios of boric acid and sodium tetraborate. Care was taken to maintain a constant ionic strength of the buffers at different pH to allow the comparison of migration times of different electropherograms. For example, to prepare the buffer at pH= 9.2 only sodium borate was used and for pH= 8.0, 62.57 mM boric acid was

mixed with 4.36 mM sodium borate. All solutions were filtered using a 0.45 μm poly syringe filter. Standard solutions of dyes ($1000\text{ }\mu\text{g ml}^{-1}$) were prepared by dissolving 10 mg of powder in 10 ml dimethyl sulfoxide (DMSO) and diluted as required to 0.2-10 $\mu\text{g ml}^{-1}$ with buffer. An organic solvent was also added to the borate buffer to enhance peak shape. Methanol and acetonitrile were explored as the organic modifier. All solutions were centrifuged prior to CE separation.

3.4 Separation properties

Experiments were carried out to examine the effect of pH, modifier and voltage on separation of the mixture of anthraquinones and flavonoids.

3.4.1 Buffer pH studies

As mentioned in Chapter 2, pH conditions can have dramatic effects on the separation of historical dyes in CE. In this study the separation parameters were investigated to achieve a promising separation of a mixture of selected anthraquinones and flavonoids using 20 mM borate buffer with 20 mM SDS. **Figure 3.1** shows the separation of 10 dyes at pH= 6.5, 7.0, 7.5, and 8.0. In these separations, under all pH conditions the dyes eluted in 2 zones: I and II where anthraquinones eluted mostly in zone I and flavonoids in zone II. This can be due to the special conformation of flavonoids where their C ring is not planar which reduces the attraction of eluents with micelles and increases their solubility in water resulting in longer migration time [34]. It can be seen that in the acidic and neutral pH values (6.5 and 7.0), Zone (I) is overlapped peaks of anthraquinone dyes and by increasing the pH at 7.5 and especially at 8.0, the

dyes eluted individually.

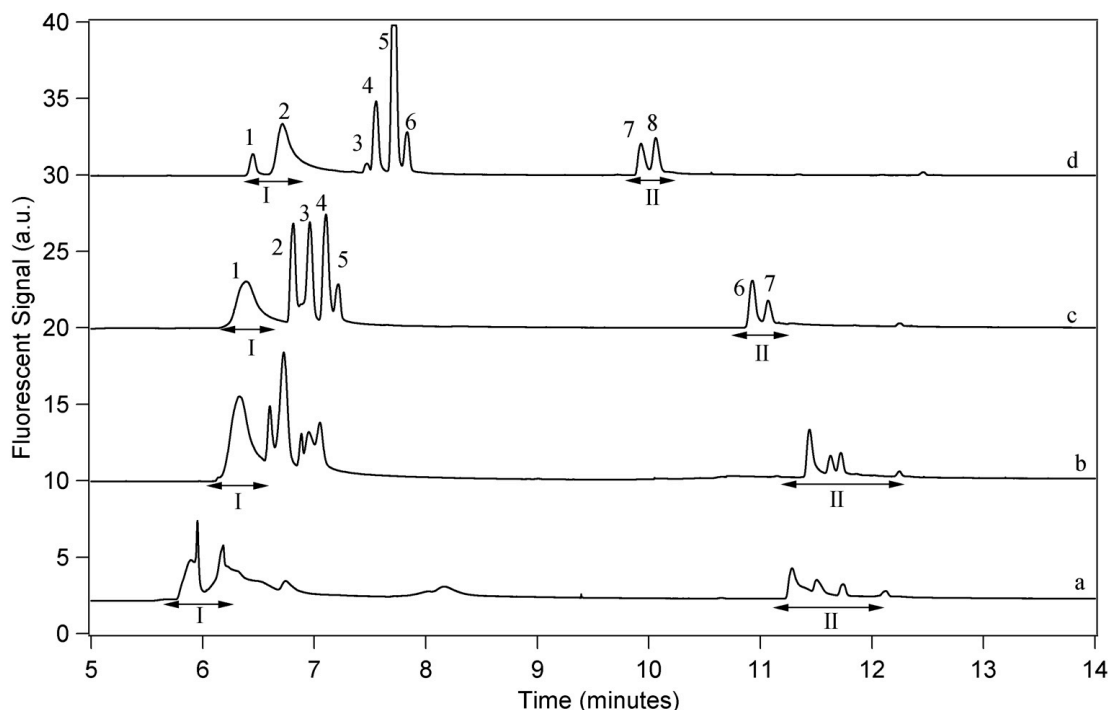


Figure 3.1 Separation of selected anthraquinones and flavonoid dyes in 20 mM borate buffer and 20 mM SDS at pH values: **(a)** 6.5, **(b)** 7.0, **(c)** 7.5, and **(d)** 8.0 using an applied potential of 18 kV. The assigned peaks for pH 8:0 are as follows: (1) $3 \mu\text{g ml}^{-1}$ alizarin, (2) $3 \mu\text{g ml}^{-1}$ purpurin, (3) $1 \mu\text{g ml}^{-1}$ emodin, (4) $2 \mu\text{g ml}^{-1}$ myricetin and $10 \mu\text{g ml}^{-1}$ carmine, (5) $1 \mu\text{g ml}^{-1}$ quercetin and $0.5 \mu\text{g ml}^{-1}$ morin, (6) $2 \mu\text{g ml}^{-1}$ luteolin, (7) $0.5 \mu\text{g ml}^{-1}$ kaempferol, and (8) $1 \mu\text{g ml}^{-1}$ apigenin.

As **Figure 3.1-d** shows alizarin and purpurin could separate from each other at pH= 8.0 and the dyes could be assigned as: (1) alizarin, (2) purpurin, (3) emodin, (4) myricetin and carmine, (5) quercetin and morin, (6) luteolin, (7) kaempferol, and (8) apigenin. The peak 4 is the overlap of myricetin and carmine and peak 5 is the overlap of quercetin and morin.

Wang *et al.* studied the migration time of selected flavonoids including Kaempferol and quercetin as a function of pH (7.0- 8.8) using MEKC. It was noted that by increasing pH from 7.0 to 8.1 with 0.1 increments, the migration time of kaempferol decreased and it

eluted after quercetin. This was in agreement with our observation (**Figure 3.1- c, d** peak 7= kaempferol). However, at pH= 8.3 they co-eluted, and at pH> 8.4 the elution order reversed and kaempferol eluted prior to quercetin [34, 35].

Khaledi *et al.* showed that the migration behavior of acidic solutes (HA) in MEKC is under the influence of 2 experimental parameters: pH values and micelle concentration, which impact the retention factor (k') and mobility (μ). Retention factor (k') in MEKC is defined as the ratio of the amount of the analyte associated into micelles to that in the aqueous phase. By increasing pH, the retention factor decreased, since at higher pH values the dissociated form (A^-) is the dominate form which is negatively charged and is repelled by micelles. This is as opposed to lower pH values, where the protonated form (HA) is the dominate one showing more interaction with micelles. It was also concluded that the more hydrophobic solute has greater retention factor due to an increase in the partition coefficient into micelles. The changes of mobility in the micellar solution with pH variations did not show the same trend as retention factor did. It is dependent on the analyte type. By increasing pH, the mobility of more hydrophobic compounds (the one with bigger k' values) decreases and the differences between the mobility of undissociated and dissociated forms (HA, A^-) is much lower than for the less hydrophobic compounds. In general, mobility of the less hydrophobic analytes showed greater changes with the pH variations. It is worth noting that in the micellar media the ionization constant of acidic compounds has a shift known as apparent dissociation constant in micellar solution ($k_{a, app}$). It is related to binding constant of the dissociated and undissociated forms of analyte in the micelles as well as micelle concentration [36].

The effect of modifier and voltage were investigated to achieve the baseline separation but it was concluded that at pH= 6.5, 7.0 and 7.5 not only methanol and acetonitrile at different concentrations (5%, 10% and 20%), but also using different voltages (18, 20, 22 and 25 kV) did not improve the separation results.

This study led us to investigate the effect of higher pH values (8.0, 8.5 and 9.20) on the separation of 4 dyes belonging to the same class of dyes anthraquinones, flavones and flavonols (the subgroups of flavonoids) and the mixture of the aforementioned dyes. In general, increasing the pH had a significant effect on the more hydrophobic compounds (flavonoids) and a slight effect on the anthraquinones.

pH studies on anthraquinones

The effect of alkaline conditions (pH= 8- 9.2) on separation of the mixture of alizarin, purpurin, emodin, and carmine at $5 \mu\text{g ml}^{-1}$ are presented in **Figure 3.2**.

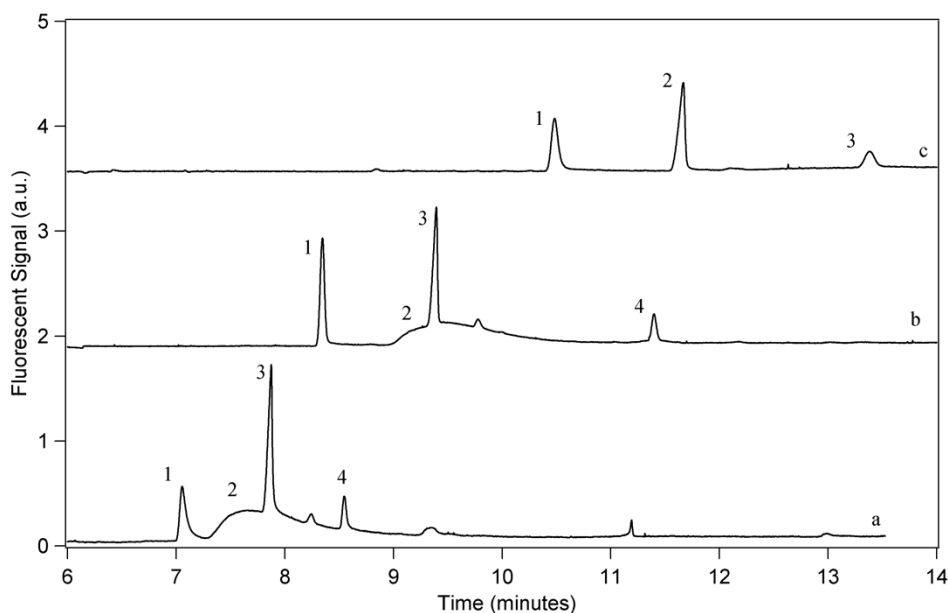


Figure 3.2 Separation of selected $5 \mu\text{g ml}^{-1}$ anthraquinones in 20 mM borate buffer and 20 mM SDS at pH values: (a) 8.0, (b) 8.5 and (c) 9.2 using an applied potential of 18 kV. The assigned peaks for pH= 8.0 and 8.5 are as follows: (1) alizarin, (2) purpurin, (3) emodin, and (4) carmine and for pH= 9.2: (1) alizarin, (2) emodin, and (3) carmine.

The comparison between the peak shapes of dyes at different pH values shows that at pH= 8.5 alizarin (1) has the sharper peak compared to the other pH conditions (**Figure 3.2-b**).

Regarding purpurin, its peak shape is less broad and sharper at pH= 8.0 but by increasing the pH it gets broader, and at pH= 9.2 it is completely flat. Concerning emodin (3) and carmine (4), they have the sharpest peaks at pH= 8.0 compared to the other pH conditions but considering the shapes of the dyes' peak the best baseline separation was achieved at pH= 8.5.

Therefore, the peaks were assigned by running experiments at pH= 8.5 and the results are summarized in **Figure 3.3**. In all runs the concentration of all dyes was $5 \mu\text{g ml}^{-1}$.

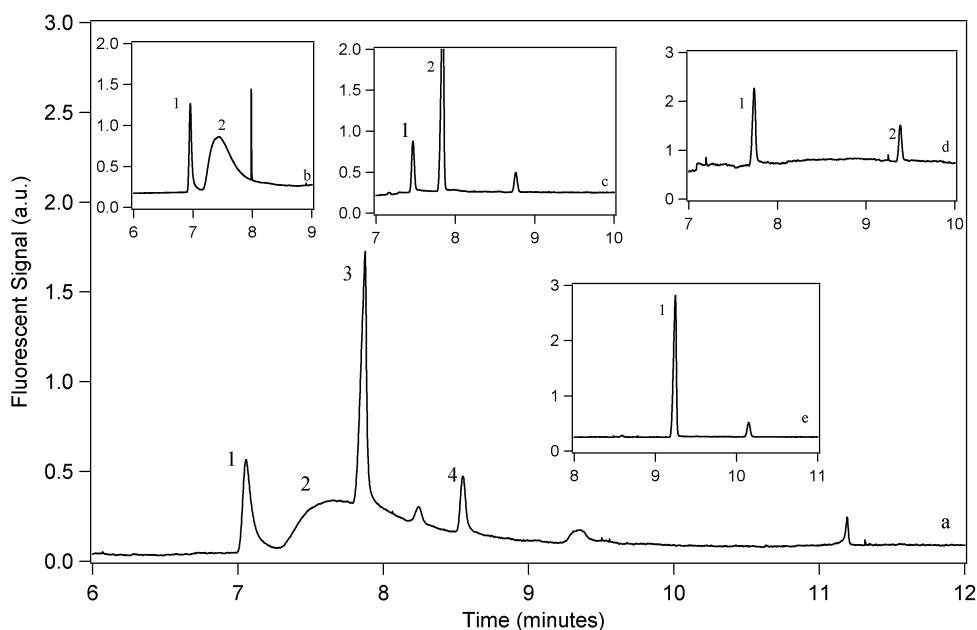


Figure 3.3 Separation of $5 \mu\text{g ml}^{-1}$ anthraquinones in 20 mM borate buffer and 20mM SDS at pH= 8.0 using 18 kV. Run **(a)**: (1) alizarin, (2) purpurin, (3) emodin and (4) carmine, **(b)**: (1) alizarin and (2) purpurin, **(c)**: (1) alizarin and (2) emodin, **(d)**: (1) alizarin and (2) carmine, and **(e)**: (1) emodin.

Figure 3.3-a shows the separation of alizarin, purpurin, emodin, and carmine. By running only alizarin and purpurin in run (b) it was confirmed that peak 1 and 2 in run (a)

belonged to alizarin and purpurin, respectively. The mixture of alizarin and emodin was injected in run (c) and peak 1 was assigned as alizarin and peak 2 as emodin. The mixture of alizarin (1) and carmine (2), were injected in run (d) and emodin was injected by itself in run (e). It can be seen that the small peak eluting at 10:20 belonged to emodin and likely can be considered as a contamination. This small peak can be identified in (a) and (c) at 8:30 and 8:50, respectively. From this study, it was concluded that the dyes eluted in run (a) were: alizarin (1), purpurin (2), emodin (3) and carmine (4).

Koyama *et al.* used MEKC (30 mM borate with 30 mM SDS at pH= 9.0 using 10% ACN; 80 mM borate at pH= 8.0) for separation of selected anthraquinones. Based on the molecules with fewer negative charges moving faster, it was concluded that the negative charges on the molecules with the OH groups at peri position with carbonyl group decreased due to hydrogen bonding. For instance, alizarin (1,2 dihydroxy anthraquinone) moves faster than (2,6 dihydroxy anthraquinone). It was also concluded that the negative charges on the molecules with ortho hydroxyl groups that form borate complex showed a longer migration times [37]. The elution order of 4 anthraquinones in our work can be explained by using this information. Carmine is the only dye that can form a borate complex and eluted last in all pH values. Regarding alizarin, purpurin and emodin it can be concluded that alizarin with 2 OH groups has fewer negative charges compared to purpurin and emodin with 3 OH groups. Therefore alizarin eluted first.

Weng *et al.* used 30 mM borate buffer at pH= 10.56 with 10% ACN and an applied voltage of 23 kV for separation of alizarin, purpurin and emodin. The elution order in this work is in agreement with our findings. Unexpectedly, as opposed to our finding that purpurin is not stable at pH above 8.5, the peak that Weng *et al.* reported was very sharp [38].

Borate buffer as well as 20 mM ammonium carbonate buffer at pH= 9.0 and 5 mM phosphate buffer at pH= 8.5 were used for separation of anthraquinones by Puchalska *et al.* None of these buffers could resolve purpurin and emodin peaks. The elution order for both pH values were alizarin, co-eluted purpurin and emodin, and carmine. At pH= 8.0 and 9.0 they eluted at 5:50, 5:55 and 6:50; and 6:30 6:50 and 9:30 minutes, respectively [19].

A mixture of dyes was separated using MEKC: 20 mM borate, 50 mM SDS, 1,3 diamine propane at pH= 8.5, $l = 50$ cm, $V = 20$ kV and the elution order of anthraquinones was reported as alizarin, carmine, and purpurin, 8:30, 8:60 and 9:40 minutes, respectively. It seems using 1,3 diamine propane had an impact on mobility of carmine which eluted prior to purpurin [21]. The order of elution obtained for alizarin and purpurin using 10 mM tetra borate buffer and 15 mM SDS at pH= 8.5, $V = 20$ kV, and $l = 61.5$ cm was in agreement with our data where alizarin and purpurin eluted at 7:30 and 9:30 minutes [30]. The results reported by other groups confirm that the elution order of anthraquinones is the same at MEKC and CZE [38], [23, 39].

pH studies on Flavones

The effect of alkaline pH on separation of the mixture of apigenin, ap-7-*O*-glu, luteolin, and lu-7-*O*-glu, are presented in **Figure 3.4**.

Under all conditions O-glycosides luteolin and apigenin (peaks 1 and 2) eluted prior to aglycones (peaks 3 and 4), since glycosylation decreases migration time owing to increasing polarity [34], [40], [41].

At pH= 8.0 and 8.5 lu-7-*O*-glu (1) eluted prior to ap-7-*O*-glu (2) with about 2 and 1 minute difference, respectively. By reaching to pH= 9.2 (c) ap-7-*O*-glu (1) eluted prior to

lu-7-*O*-glu (2). The elution order of apigenin and luteolin was shifting by changing pH as: luteolin eluted prior to apigenin at pH = 8.5 and 9.2 and after at pH= 8.0.

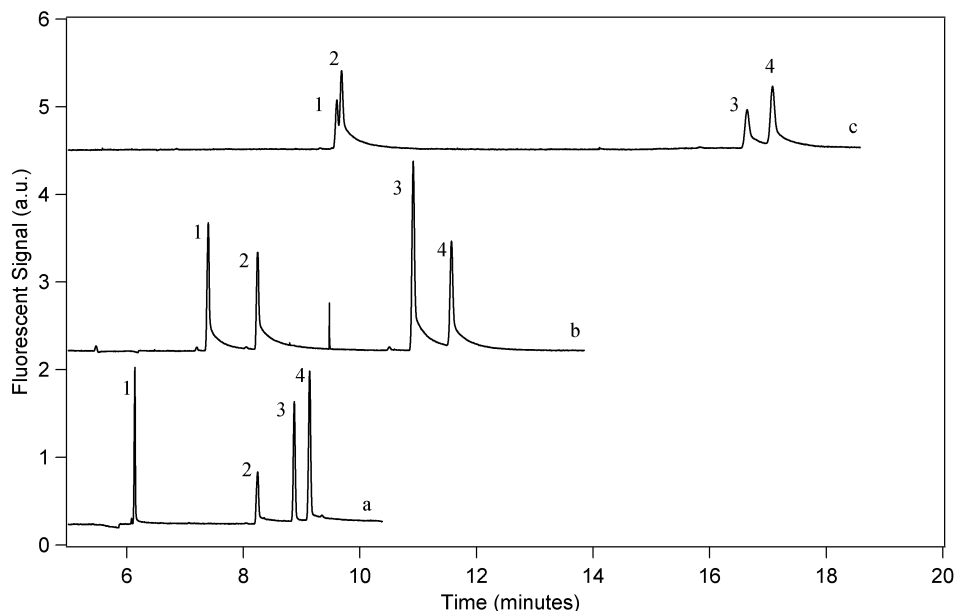


Figure 3.4 Separation of selected flavones in 20 mM borate buffer and 20 mM SDS at pH values: **(a)** 8.0, **(b)** 8.5 and **(c)** 9.2 using an applied potential of 18 kV. The assigned peaks in **(a)**: (1) lu-7-*O*-glu, (2) ap-7-*O*-glu, (3) luteolin, and (4) apigenin. Run **(b)**: (1) lu-7-*O*-glu, (2) ap-7-*O*-glu, (3) apigenin, and (4) luteolin. In run **(c)**: (1) ap-7-*O*-glu, (2) lu-7-*O*-glu, (3) luteolin, and (4) apigenin. All dyes were 5 $\mu\text{g ml}^{-1}$ except for apigenin which was 0.5 $\mu\text{g ml}^{-1}$.

Wang *et al.* studied the changes in mobility of luteolin and apigenin as a function of pH in the range of pH= 8.8 to 9.3 with 0.1 increments. The results from this study showed that by increasing the pH from 8.8 to 9.2 the mobility of apigenin decreased and the mobility of luteolin increased, and both dyes showed the same mobility between pH 9.1 and 9.2 [42]. By increasing the pH, at pH =9.3 the mobility of apigenin did not change while luteolin showed an increase in the mobility. One can comment that the difference in the elution order of these 2 dyes can be due to the formation of borate complex by luteolin but Markham studied the effect of pK_a on electrophoretic mobility of mono and di hydroxyl flavones in borate buffer at pH= 9.5 and it was concluded that in the case of mono hydroxyl

flavones, mobility decreases with an increase in pK_a . The addition of the second hydroxyl group in the meta and para di hydroxyl flavones showed a minor influence on the pK_a of mono hydroxyl compound with no obvious change in mobility. However, it was noticed that the mobility changed significantly when the second hydroxyl was introduced in the ortho position. The UV absorption study of 7,8-dihydroxyl flavones and 3',4'-dihydroxyl flavones (luteolin) in borate solution showed a bathochromic shift in comparison to HCl-Tris solution indicating the formation of borate complex. In the case of other di hydroxyl flavones such as 2',3'- dihydroxyl flavone there was no changes in UV spectrum indicating that the borate complex did not form. The formation of borate complex depends on the acidity of the hydroxyl groups in alkaline pH; the more acidic hydroxyl groups showed a higher level of ionization which probably led to a higher amount of selective complex formation [43].

From this study, it can be concluded that the best separation was achieved at pH= 8.5. In order to assign the dyes' peaks at pH= 9.2, different concentrations of dyes were used and the results are shown in **Figure 3.5**. A mixture of 4 dyes at $5 \mu\text{g ml}^{-1}$ except for apigenin which was $0.5 \mu\text{g ml}^{-1}$ was injected in run 'a'. By increasing the concentration of lu-7-*O*-glu to $7 \mu\text{g ml}^{-1}$ in run (b) there was an overlap between this dye and ap-7-*O*-glu (peak 1). In this run the concentration of luteolin was reduced from 5 to $2 \mu\text{g ml}^{-1}$ and it indicated that the last peak in run (b) belonged to luteolin. A small increase in amount of lu-7-*O*-glu in run (c), increased the size of peak 2.

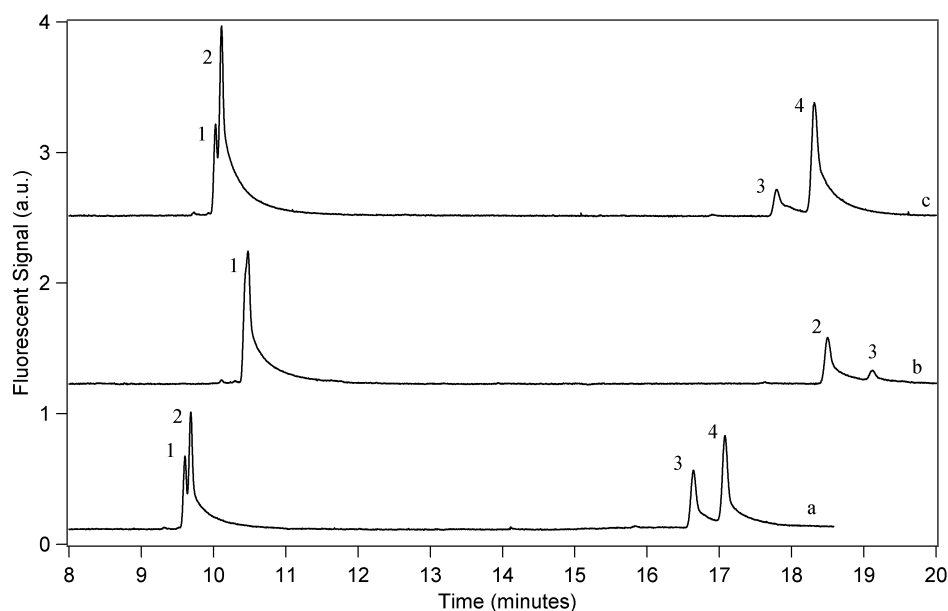


Figure 3.5 Separation of selected flavones in 20 mM borate buffer and 20mM SDS at pH= 9.2 using an applied potential of 18 kV. The assigned peaks in **(a)** are: (1) $5 \mu\text{g ml}^{-1}$ ap-7-*O*-glu, (2) $5 \mu\text{g ml}^{-1}$ lu-7-*O*-glu, (3) $5 \mu\text{g ml}^{-1}$ luteolin, and (4) $0.5 \mu\text{g ml}^{-1}$ apigenin, in **(b)**: (1) $5 \mu\text{g ml}^{-1}$ ap-7-o-glu and $7 \mu\text{g ml}^{-1}$ lu-7-*O*-glu, (2) $0.5 \mu\text{g ml}^{-1}$ apigenin, and (3) $2 \mu\text{g ml}^{-1}$ luteolin, in **(c)**: (1) $5 \mu\text{g ml}^{-1}$ ap-7-*O*-glu, (2) $10 \mu\text{g ml}^{-1}$ lu-7-*O*-glu, (3) $2.5 \mu\text{g ml}^{-1}$ luteolin, and (4) $0.7 \mu\text{g ml}^{-1}$ apigenin.

Also, decreasing the amount of luteolin and increasing the amount of apigenin resulted in a smaller peak for luteolin and bigger peak for apigenin. It proved that peaks 3 and 4 belonged to luteolin and apigenin, respectively.

In order to assign the apigenin peak at pH= 8.0 several experiments were performed and the results are summarized in **Figure 3.6**. Peak 4 in run (a) was assigned as apigenin with a concentration of $0.5 \mu\text{g ml}^{-1}$. In following runs the concentration of all dyes were the same as used in run (a) except for luteolin which was reduced to $2.5 \mu\text{g ml}^{-1}$ in run (b) and increased to $8 \mu\text{g ml}^{-1}$ in run (c).

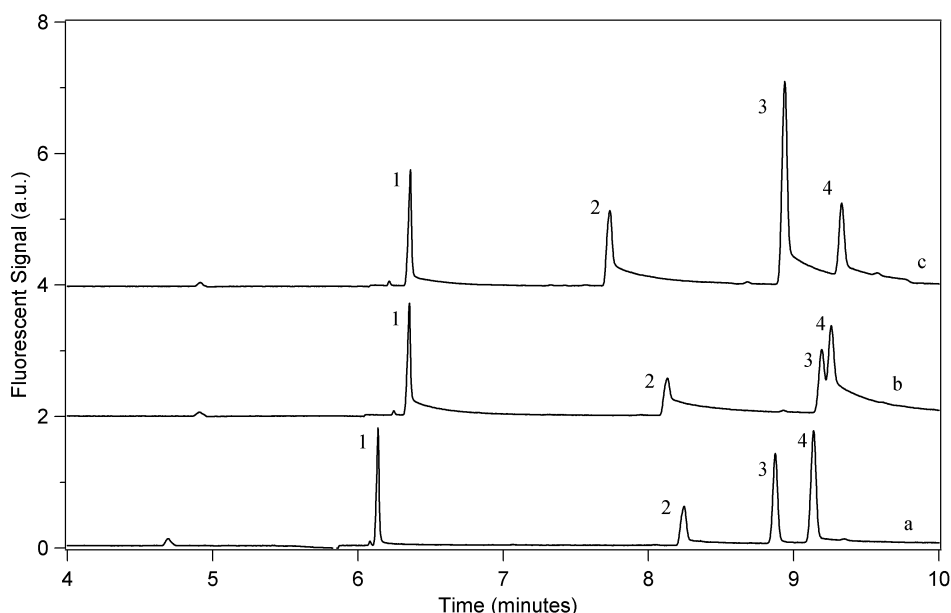


Figure 3.6 Separation of selected flavones in 20 mM borate buffer and 20 mM SDS at pH= 8.0 using an applied potential of 18 kV. The assigned peaks in **(a)** are: (1) $5 \mu\text{g ml}^{-1}$ lu-7-*O*-glu, (2) $5 \mu\text{g ml}^{-1}$ ap-7-*O*-glu, (3) $5 \mu\text{g ml}^{-1}$ luteolin, and (4) $0.5 \mu\text{g ml}^{-1}$ apigenin, and in **(b)**: (1) $5 \mu\text{g ml}^{-1}$ lu-7-*O*-glu, (2) $5 \mu\text{g ml}^{-1}$ ap-7-*O*-glu, (3) $2.5 \mu\text{g ml}^{-1}$ luteolin, and (4) $5 \mu\text{g ml}^{-1}$ apigenin, in **(c)**: (1) $5 \mu\text{g ml}^{-1}$ lu-7-*O*-glu, (2) $10 \mu\text{g ml}^{-1}$ ap-7-*O*-glu, (3) $8 \mu\text{g ml}^{-1}$ luteolin, and (4) $5 \mu\text{g ml}^{-1}$ apigenin.

The changes in peak size were compared with the results in run (a) indicating that only peak 3 had reduced in run (b) and increased in run (c).

pH studies on flavonols

The effect of alkaline pH (8.0- 9.2) on separation of the mixture of ($5 \mu\text{g ml}^{-1}$) kaempferol, myricetin, quercetin, and morin is presented in **Figure 3.7**. The comparison between the separations of dyes at different pH conditions shows that at pH= 8.0 all dyes eluted in less than one minute and the last 2 dyes overlapped with each other (quercetin and morin). By increasing the pH, elution time increased e.g. at pH= 9.2 the dyes eluted in 22 minutes. By compromising the run time and the best baseline separation, it can be concluded that the best pH for separation of flavonols was pH= 8.5 (**Figure 3.7-b**).

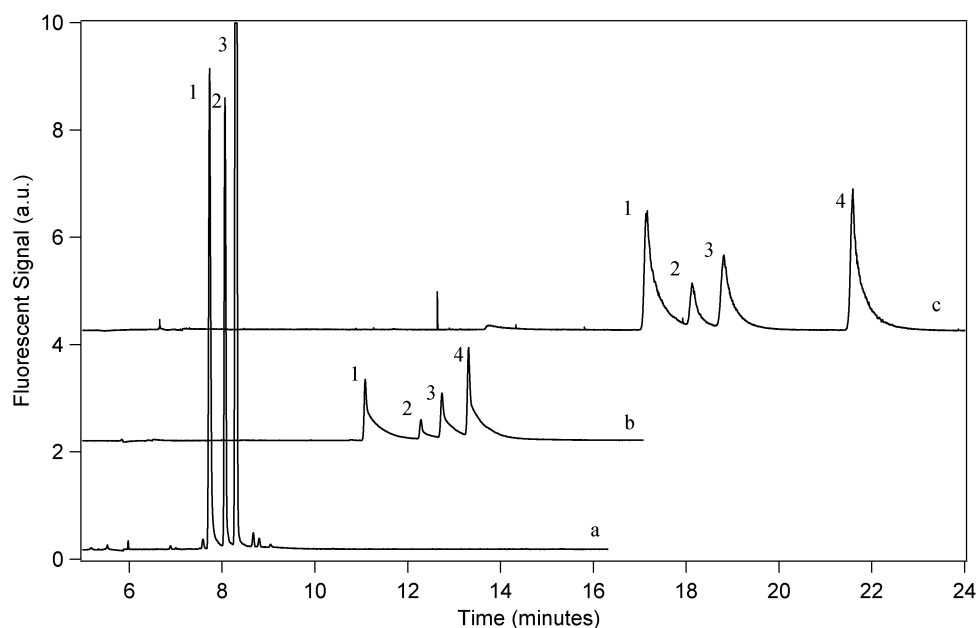


Figure 3.7 Separation of selected flavonols in 20 mM borate buffer and 20 mM SDS at pH values: **(a)** 8.0, **(b)** 8.5 and **(c)** 9.2 using an applied potential of 18 kV. The assigned peaks in **(a)**: (1) kaempferol, (2) myricetin, (3) quercetin, and morin. In **(b)** and **(c)**: (1) kaempferol, (2) myricetin, (3) quercetin, and (4) morin. All dyes were $5 \mu\text{g ml}^{-1}$.

It was observed that the retention times for these dyes appeared to change under different pH conditions. This is likely a function of the slight differences in the pK_{a} values of these dyes which are similar in magnitude to these pH values. For example, the $\text{pK}_{\text{a}2}$ values have been reported in the literature for morin, kaempferol, and quercetin are 8.6, 9.0, and 9.4 respectively [44]. Unfortunately, pK_{a} values for myricetin using CE have not been reported in the literature and other research groups have reported that pK_{a} values can be quite different from these values depending on how they were determined [45]. Furthermore, pK_{a} values for myricetin are difficult to determine due to the poor stability of this dye [45]. Therefore, to be sure of the identity for each peak, under different pH conditions, solutions containing a single dye or spiked solutions of dyes were injected and the retention times for each dye were compared to the mixture. In order to assign the peaks at pH= 8.0,

first a mixture of kaempferol, myricetin, quercetin, and morin at $5 \mu\text{g ml}^{-1}$ was injected (**Figure 3.8-a**). A mixture of dyes was injected in run (b) and the concentration of all dyes was reduced by a fifth ($1 \mu\text{g ml}^{-1}$) except for kaempferol which was reduced by a tenth ($0.5 \mu\text{g ml}^{-1}$). The first and smallest eluting peak in run (b) belonged to kaempferol.

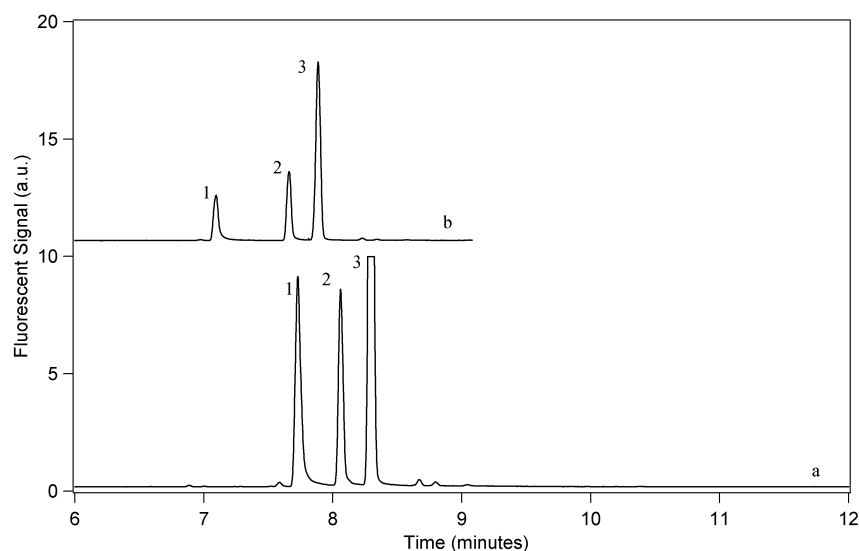


Figure 3.8 Separation of selected flavonols in 20 mM borate buffer and 20 mM SDS at pH= 8.0 using an applied potential of 18 kV. The assigned peaks in **(a)** and **(b)** are: (1) kaempferol, (2) myricetin, (3) quercetin and morin. In run **(a)** the concentrations of all dyes were $5 \mu\text{g ml}^{-1}$ and in run **(b)** they were $1 \mu\text{g ml}^{-1}$ except for kaempferol which was $0.5 \mu\text{g ml}^{-1}$.

Figure 3.9 shows different separations for identification of flavonols at pH= 8.5. A mixture of quercetin (1) and morin (2) were injected in run (a). Kaempferol and myricetin were added in run (b) and (c), respectively. The comparison of the elution times showed that peak (1) in both runs (b and c) belonged to kaempferol and peak (2) in run (c) belonged to myricetin.

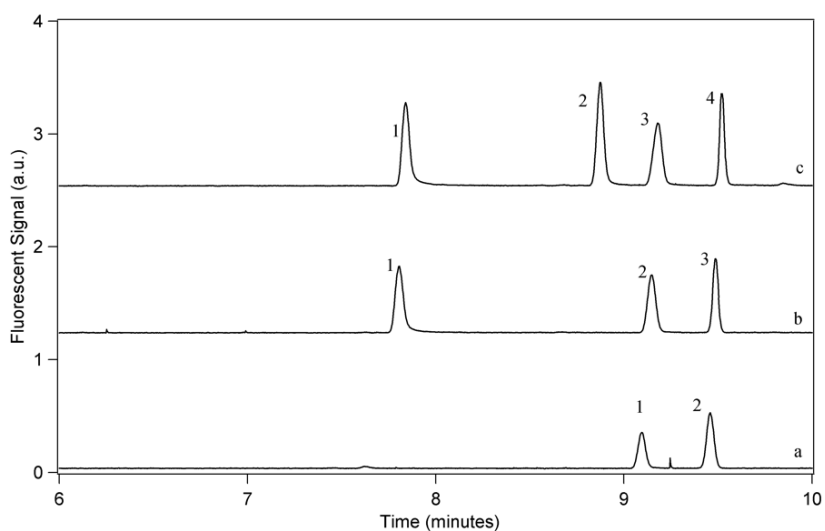


Figure 3.9 Separation of a mixture of flavonols in a 20 mM borate buffer and 20 mM SDS at pH= 8.5 using an applied potential of 18 kV. Peak identities: Run **(a)**: (1) $1 \mu\text{g ml}^{-1}$ quercetin, (2) $2 \mu\text{g ml}^{-1}$ morin. Run **(b)**: (1) $1 \mu\text{g ml}^{-1}$ kaempferol, (2) $1 \mu\text{g ml}^{-1}$ quercetin, (3) $2 \mu\text{g ml}^{-1}$ morin. Run **(c)**: (1) $1 \mu\text{g ml}^{-1}$ kaempferol, (2) $4 \mu\text{g ml}^{-1}$ myricetin, (3) $1 \mu\text{g ml}^{-1}$ quercetin, (4) $2 \mu\text{g ml}^{-1}$ morin.

In order to identify the peaks of flavonols at pH= 9.2 a different mixture of the dyes at $5 \mu\text{g ml}^{-1}$ was injected **Figure 3.10-a**.

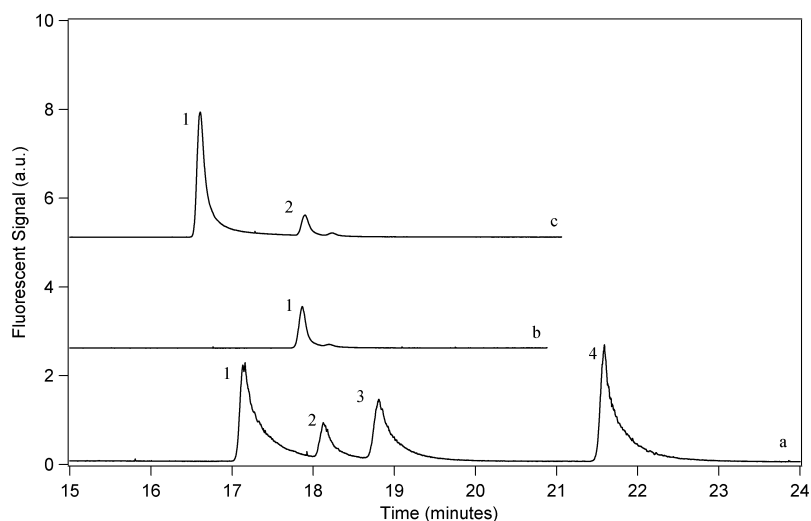


Figure 3.10 Separation of selected flavonols in 20 mM borate buffer and 20 mM SDS at pH= 8.0 using an applied potential of 18 kV. The concentration of dyes was $5 \mu\text{g ml}^{-1}$ in all runs. The assigned peaks in **(a)**: (1) kaempferol, (2) myricetin, (3) quercetin, and (4) morin. In run **(b)**: (1) myricetin and in **(c)**: (1) kaempferol and (2) myricetin.

Myricetin was injected as a single dye in run (b) helping to identify peaks (1) and (2) in both runs (a) and (c) as kaempferol and myricetin, respectively.

Based on this preliminary research the elution order of each class of dye at pH= 8.0, 8.5 and 9.2 was determined. This data can be combined to help understand the elution order of a mixture of dyes from different classes of dyes under different pH conditions. Therefore, from **Figures 3.2, 3.4 and 3.7** the following elution orders can be predicted:

pH= 8.0: lu-7-*O*-glu, alizarin, purpurin, kaempferol, emodin, myricetin, ap-7-*O*-glu, (quercetin + morin), carmine, luteolin and apigenin.

pH= 8.5: lu-7-*O*-glu, alizarin, ap-7-*O*-glu, purpurin, emodin, (kaempferol + apigenin), carmine, luteolin, myricetin, quercetin, and morin.

pH= 9.2: lu-7-*O*-glu, ap-7-*O*-glu, alizarin, emodin, carmine, luteolin, (kaempferol + apigenin), myricetin, quercetin and morin.

3.4.2 The effect of Modifier on separation at pH= 8.0

The addition of organic reagents to the buffer affects viscosity, zeta potential, dielectric constant and the electroosmotic flow (EOF) [46]. Reducing the EOF can help to resolve analytes that have small differences in electrophoretic mobility [47]. **Figure 3.1** showed that the quality of separation of a mixture of dyes at pH= 8.0 (run d) was better than acidic pH conditions. However, at this pH myricetin overlapped with carmine (peak 4) and quercetin overlapped with morin (peak 5). Adding an organic modifier was also a parameter that changed the quality of separation. Separations that were carried out in the absence of an organic modifier were generally not successful. McGhie *et al.* [48] examined the effects

of methanol on the migration order of a synthetic mixture of flavonoids that included apigenin. These authors concluded that 20% methanol gave the best separation.

In this research, acetonitrile and methanol were used as modifiers for separating a mixture of dyes at pH= 8.0 using 18 kV. The results are presented in **Figure 3.11** and **Figure 3.12**. For each run a fresh solution of the same mixture of the dyes was injected and by adding the modifier at 5, 10 and 20 % (v/v) the quality of separations was explored. It should be mentioned in this work as opposed to McGhie et. al's finding [48], using 20% of either acetonitrile or methanol did not improve the separation. Since the results were not promising they are not presented.

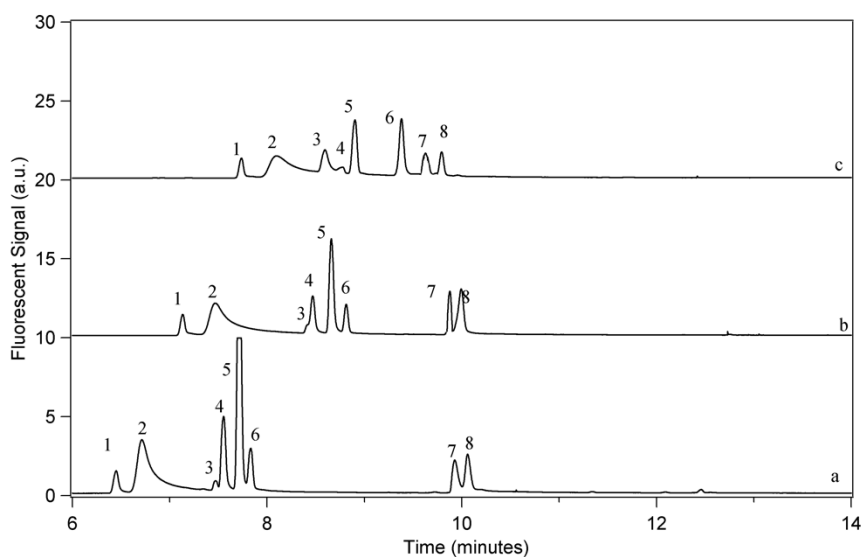


Figure 3.11 Separation of selected anthraquinones and flavonoid dyes ($3 \mu\text{g ml}^{-1}$ alizarin, $3 \mu\text{g ml}^{-1}$ purpurin, $1 \mu\text{g ml}^{-1}$ emodin, $2 \mu\text{g ml}^{-1}$ myricetin, $10 \mu\text{g ml}^{-1}$ carmine, $1 \mu\text{g ml}^{-1}$ quercetin, $0.5 \mu\text{g ml}^{-1}$ morin, $2 \mu\text{g ml}^{-1}$ luteolin, $0.5 \mu\text{g ml}^{-1}$ kaempferol and $1 \mu\text{g ml}^{-1}$ apigenin in a 20 mM borate buffer and 20 mM SDS at pH= 8.0: **(a)** no modifier, **(b)** in 5% (v/v), and **(c)** in 10% (v/v) of acetonitrile at 18 kV. The assigned peaks for **(a)** and **(b)** are: (1) alizarin, (2) purpurin, (3) emodin, (4) myricetin and carmine, (5) quercetin and morin, (6) luteolin, (7) kaempferol, and (8) apigenin for **(c)** are the same as in (a) and (b), except for (3) emodin and myricetin, (4) carmine.

The findings from identification runs (these results are presented in following section) showed that peak 4 in **Figure 3.11-a** was the overlap of myricetin and carmine, and peak

5 was the overlap of quercetin and morin. In order to resolve the peaks, acetonitrile was added to the mixture at 5% and 10% (v/v), in run (b) and (c), respectively. It was concluded that at pH= 8.0 different concentrations of acetonitrile did not improve separation of these peaks. Also, by increasing the amount of acetonitrile the elution time for most of the dyes was increased except for kaempferol and apigenin, which eluted about the same time as in run (a). In the higher concentration of acetonitrile run (c), myricetin eluted at the same time as emodin. It was concluded that peak (3) was an overlap of these 2 dyes and peak (4) was carmine.

The effect of methanol as a modifier is shown in **Figure 3.12**, where run (a) was without modifier, and run (b) had 5% and (c) had 10% (v/v) of methanol, respectively.

This data confirmed that methanol, the same as acetonitrile did not improve resolution of the peaks. By increasing the amount of methanol the same as acetonitrile the elution time of most dyes increased except for kaempferol and apigenin. Also, by increasing methanol, fluorescence properties of most dyes, especially kaempferol decreased. As opposed to acetonitrile, methanol did not have any impact on the elution time of myricetin. Therefore, the elution order of dyes in all runs were the same. Wang *et al.* noted that by adding the modifier, if the migration time of the eluents increased it was due to the dominant impact of modifier on decreasing electroosmotic flow. If the migration time of the eluents decreased it was due to the dominant impact of the modifier on the interactions between eluents and micelles [35].

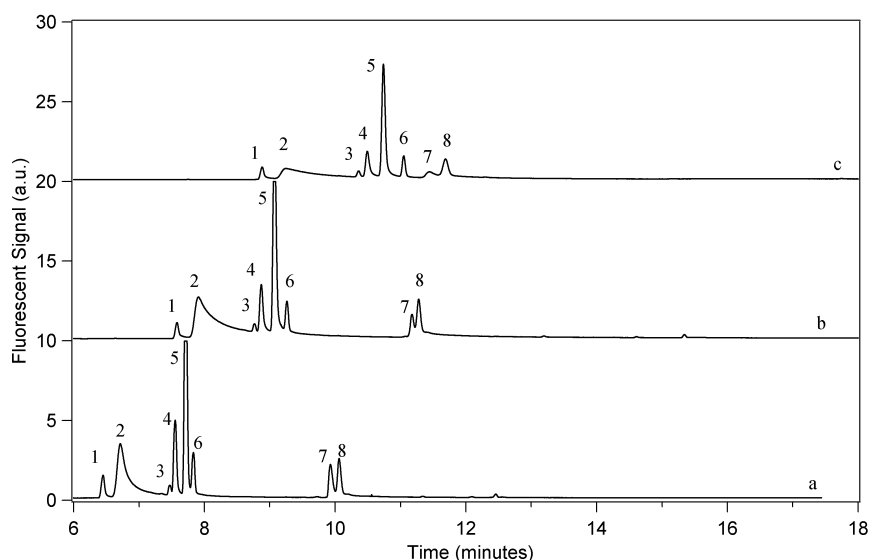


Figure 3.12 Separation of selected anthraquinones and flavonoid dyes ($3 \mu\text{g ml}^{-1}$ alizarin, $3 \mu\text{g ml}^{-1}$ purpurin, $1 \mu\text{g ml}^{-1}$ emodin, $2 \mu\text{g ml}^{-1}$ myricetin, $10 \mu\text{g ml}^{-1}$ carmine, $1 \mu\text{g ml}^{-1}$ quercetin, $0.5 \mu\text{g ml}^{-1}$ morin, $2 \mu\text{g ml}^{-1}$ luteolin, $0.5 \mu\text{g ml}^{-1}$ kaempferol) in a 20 mM borate buffer and 20 mM SDS at pH= 8.0: **(a)** no modifier, **(b)** in 5% (v/v), and **(c)** in 10% (v/v) of methanol at 18 kV. The assigned peaks for **(a)**, **(b)** and **(c)** are: (1) alizarin, (2) purpurin, (3) emodin, (4) myricetin and carmine, (5) quercetin and morin, (6) luteolin, (7) kaempferol, and (8) apigenin.

3.4.3 The effect of voltage on separation at pH= 8.0

Experiments were carried out to examine the effect of applied voltage (18-25 kV) on the separation of these dyes without modifier and using 5% acetonitrile or 5% methanol. **Figure 3.13** shows the separation of the mixture of aforementioned dyes at different voltages without modifier. Figure 3.14 and 3.15 show the effect of voltage on separation in the presence of 5% acetonitrile and 5% methanol, respectively. It should be mentioned in Figures 3.13 and 3.14 (runs: a, b and d) peak (8) before kaempferol (peak 9) is likely the impurity which was from emodin solution. **Figure 3.13** indicated that by increasing the voltage the elution time decreased but it did not help to resolve the overlapping peaks (peak 6 and 7).

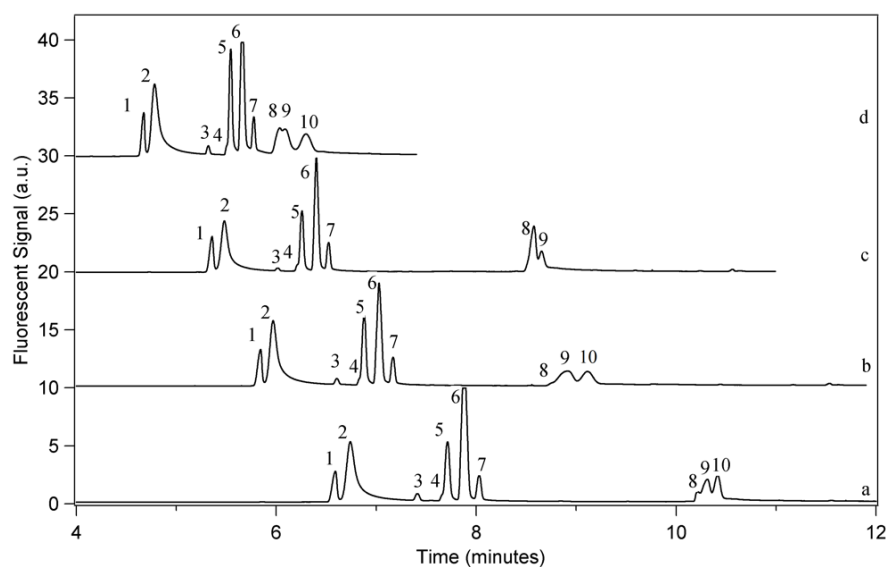


Figure 3.13 Separation of selected anthraquinones and flavonoid dyes ($3 \mu\text{g ml}^{-1}$ alizarin, $3 \mu\text{g ml}^{-1}$ purpurin, $1 \mu\text{g ml}^{-1}$ emodin, $2 \mu\text{g ml}^{-1}$ myricetin, $10 \mu\text{g ml}^{-1}$ carmine, $1 \mu\text{g ml}^{-1}$ quercetin, $0.5 \mu\text{g ml}^{-1}$ morin, $2 \mu\text{g ml}^{-1}$ luteolin, $0.5 \mu\text{g ml}^{-1}$ kaempferol) in a 20 mM borate buffer and 20 mM SDS at pH= 8.0, at: **(a)** 18 kV, **(b)** 20 kV, **(c)** 22 kV and **(d)** 25 kV. The assigned peaks for **(a)**, **(b)** and **(d)** are: (1) alizarin, (2) purpurin, (3) emodin, (4) carmine, (5) myricetin, (6) quercetin and morin, (7) luteolin, (8) the impurity from emodin, (9) kaempferol, and (10) apigenin. In run **(c)** the peaks (1 to 7) are the same as assigned in other runs except peak (8) is kaempferol, and (9) is apigenin. Reproduced with permission from Ref [49]. – Copyright: MDPI AG

The same results were concluded from **Figures 3.14 and 3.15**. They show that using different voltages in the presence of modifiers at pH= 8.0 could not improve the baseline separation of myricetin and carmine as well as quercetin and morin. Using 5% methanol at 20 kV (**Figure 3.15 -b**) had a minor impact on resolving the peaks. As it shows the peak belonging to quercetin eluted as a shoulder between myricetin and morin.

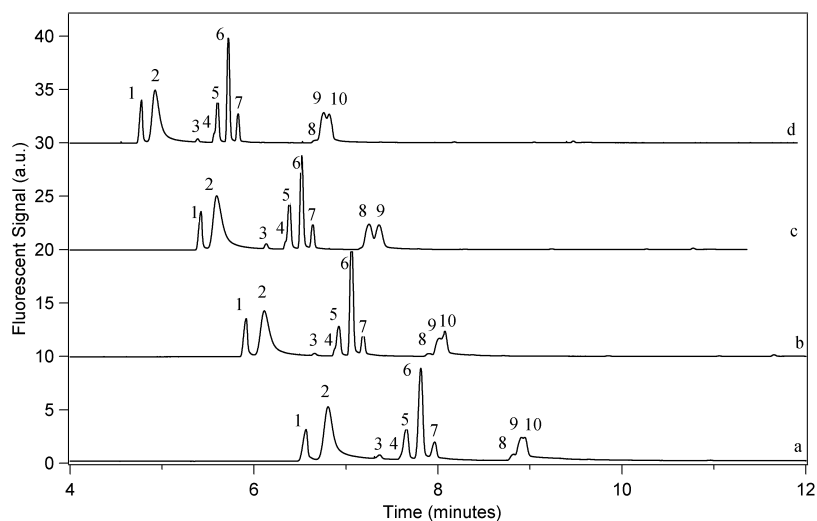


Figure 3.14 Separation of selected anthraquinones and flavonoid dyes (as mentioned in Figure 3.13) in presence 5% (v/v) of acetonitrile in a 20 mM borate buffer and 20 mM SDS at pH= 8.0, at: **(a)** 18kV, **(b)** 20 kV, **(c)** 22 kV and **(d)** 25 kV. The assigned peaks for **(a)**, **(b)** and **(d)** are: (1) alizarin, (2) purpurin, (3) emodin, (4) carmine, (5) myricetin, (6) quercetin and morin, (7) luteolin, (8) the impurity from emodin, (9) kaempferol, and (10) apigenin. In run **(c)** the peaks are the same as assigned in other runs except peak (8) is kaempferol, and (9) is apigenin. (d) Adapted from Ref. [26] – Copyright: Elsevier

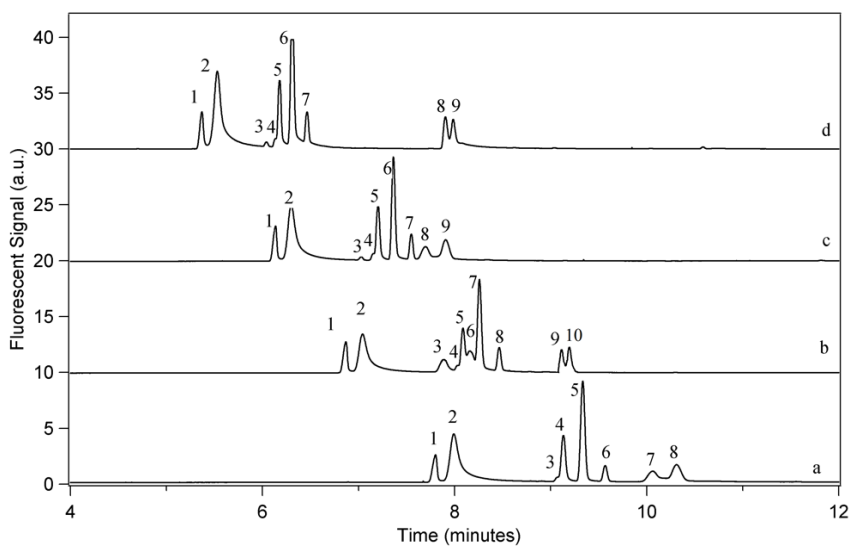


Figure 3.15 Separation of selected anthraquinones and flavonoid dyes (as mentioned in Figure 3.13) in presence 5% (v/v) of methanol in a 20 mM borate buffer and 20 mM SDS at pH= 8.0: **(a)** 18kV, **(b)** 20 kV, **(c)** 22 kV and **(d)** 25 kV. The assigned peaks for **(a)**: (1) alizarin, (2) purpurin, (3) emodin, (4) carmine + myricetin, (5) quercetin and morin, (6) luteolin, (7) kaempferol, and (8) apigenin. In **(b)** the peaks are: (1) alizarin, (2) purpurin, (3) emodin, (4) carmine (5) myricetin, (6) quercetin, (7) morin, (8) luteolin, (9) kaempferol, and (10) apigenin. In runs **(c)** and **(d)** the peaks assigned as: (1) alizarin, (2) purpurin, (3) emodin, (4) carmine, (5) myricetin, (6) quercetin and morin, (7) luteolin, (8) kaempferol, and (9) apigenin.

3.4.4 Identification of dyes at pH= 8.0

In order to assign fluorescence peaks to specific dyes, the elution times of individual dyes were determined separately, prior to separating mixtures. To confirm the peak identity, the elution time of each dye was also determined relative to the other dyes by successively adding to or removing dyes from a known mixture. In order to identify the peaks at pH= 8.0 small groups of dyes were injected and the peaks were compared. The results are given in **Figures 3.16** and **3.17**.

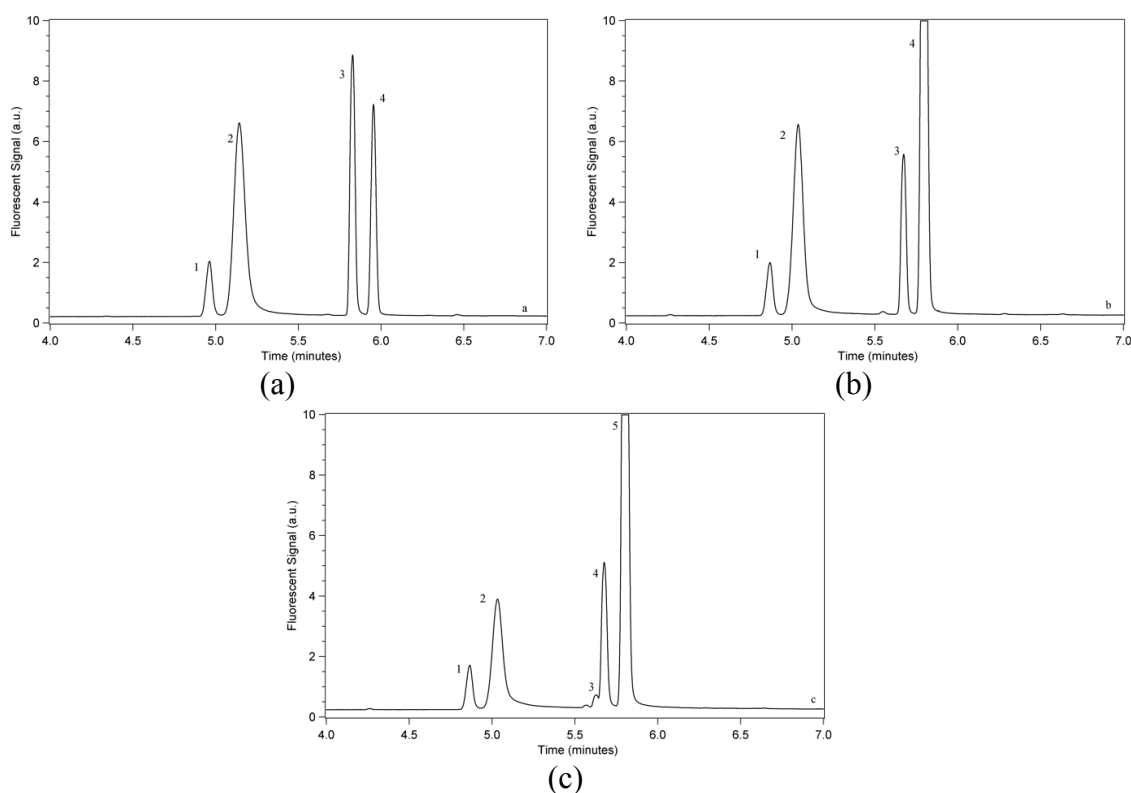


Figure 3.16 Separation of selected anthraquinones and flavonoid dyes in a 20 mM borate buffer and 20 mM SDS at pH= 8.0 using 20 kV. In run **(a)** peak identities: (1) alizarin, (2) purpurin, (3) myricetin, (4) and morin. Run **(b)** the peaks are: (1) alizarin, (2) purpurin, (3) myricetin, (4) quercetin + morin. Run **(c)** the peaks are assigned as: (1) alizarin, (2) purpurin, (3) carmine, (4) myricetin, (5) quercetin + morin.

A mixture of (1) $5 \mu\text{g ml}^{-1}$ alizarin, (2) $3 \mu\text{g ml}^{-1}$ purpurin, (3) $2 \mu\text{g ml}^{-1}$ myricetin, and (4) $0.2 \mu\text{g ml}^{-1}$ morin was injected in run (a) and the baseline separation was achieved.

The same mixture of dyes with the same concentration used in run (a) was prepared for run (b), including $2\ \mu\text{g ml}^{-1}$ quercetin. This run showed that peak (4) had an increase which is evidence of overlapping of quercetin and morin. Run (c) shows the mixture of dyes used in run (b) including $10\ \mu\text{g ml}^{-1}$ carmine, which eluted as a shoulder peak of myricetin (peak 4).

From this experiment elution order of dyes was defined as: alizarin, purpurin, carmine, myricetin, and overlapping of quercetin and morin. In the next study the peaks were assigned successively by removing dyes from a known mixture. The results are presented in **Figure 3.17(a-i)**.

Figure 3.17-a: a mixture of ten dyes

In **Figure 3.17-a** a mixture of anthraquinones and flavonoid dyes were injected and the peaks were assigned a: (1) alizarin, (2) purpurin, (3) emodin, (4) myricetin and carmine, (5) quercetin and morin, (6) luteolin, (7) kaempferol, and (8) apigenin. It was hypothesized that peak (4) is the overlap of myricetin and carmine and peak (5) is the overlap of quercetin and morin. In the following runs 2 or 3 dyes were removed from run (a).

Figure 3.17-b: run (a) without quercetin (5) and luteolin (6)

In **Figure 3.17-b** the same mixture of dyes used in run (a) was prepared excluding quercetin and luteolin. The comparison between these runs, showed that peak (5) in this run is smaller than peak (5) eluted in run (a) and belonged to morin. By removing quercetin, it was proved that peak (5) was an overlap of quercetin and morin in run (a). Since luteolin was excluded from the mixture, the comparison between run (a) and (b) shows that peak (6) in run (a) belonged to luteolin.

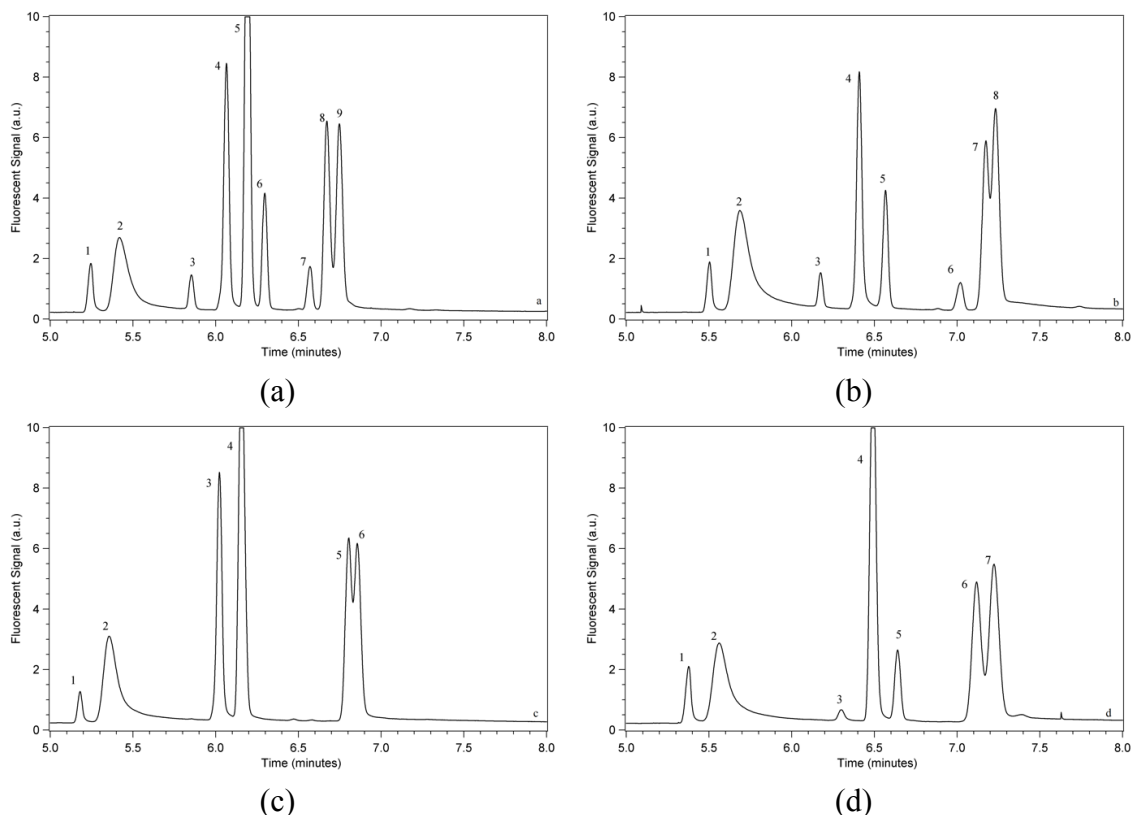


Figure 3.17 Separation of anthraquinones and flavonoid dyes in a 20 mM borate buffer and 20 mM SDS at pH= 8.0 using an applied potential of 25 kV. The concentrations of dyes were: 3 $\mu\text{g ml}^{-1}$ alizarin, 3 $\mu\text{g ml}^{-1}$ purpurin, 1 $\mu\text{g ml}^{-1}$ emodin, 2 $\mu\text{g ml}^{-1}$ myricetin, 10 $\mu\text{g ml}^{-1}$ carmine, 1 $\mu\text{g ml}^{-1}$ quercetin, 0.5 $\mu\text{g ml}^{-1}$ morin, 2 $\mu\text{g ml}^{-1}$ luteolin, 0.5 $\mu\text{g ml}^{-1}$ kaempferol, and 1 $\mu\text{g ml}^{-1}$ apigenin. Peak identities: run **(a)**: (1) alizarin, (2) purpurin, (3) emodin, (4) myricetin and carmine, (5) quercetin and morin, (6) luteolin, (7) the impurity from emodin, (8) kaempferol, and (9) apigenin. Run **(b)**: (1) alizarin, (2) purpurin, (3) emodin, (4) myricetin and carmine, (5) morin, (6) the impurity from emodin, (7) luteolin, (8) kaempferol, and (9) apigenin. Run **(c)**: (1) alizarin, (2) purpurin, (3) myricetin and carmine, (4) quercetin and morin, (5) kaempferol, and (6) apigenin. Run **(d)**: (1) alizarin, (2) purpurin, (3) carmine, (4) quercetin and morin, (5) luteolin, (6) kaempferol, and (7) apigenin.

Figure 3.17-c: run (a) without emodin (3) and luteolin (6)

In **Figure 3.17-c** emodin and luteolin were removed from the mixture used in run (a), but instead of 7 peaks, 6 peaks eluted. It was hypothesized that the third peak (peak 7 in run a) is the impurity of emodin. Therefore, peak (3) and (6) in run (a) were considered as emodin and luteolin, respectively.

Figure 3.17-d: run (a) without emodin (3) and myricetin (4)

Myricetin and emodin were excluded from the mixture of dyes used for this run.

By comparing the peaks in run (a) and (b) it was concluded that peak 3 and 4 in run (a) belonged to emodin and myricetin, respectively. Since in this run peak 7 did not show up, it was proved that peak 7 belonged to the impurity of emodin. By removing myricetin peak 4 belonging to carmine appeared. Therefore, in run (a) peak 4 was the overlap of these 2 dyes.

Figure 3.17-e: run (a) without myricetin (4) and kaempferol (8)

In this run the same mixture of dyes used in run (a) was prepared; excluding myricetin and kaempferol. The peak assigned as (4) in this run is much smaller than peak (4) in run (a). It was concluded that by removing myricetin, the peak belonging to carmine could be observed. Therefore, the hypothesis about peak (4) as an overlap peak of myricetin and carmine in run 'a' was true. Since in this run after peak (7) (the impurity peak) there is only one peak which belonged to apigenin, it can be concluded that peak (8) in first run belonged to kaempferol.

Figure 3.17-f: run (a) without carmine (4) and apigenin (9)

In this run carmine and apigenin were not in the mixture injected. The comparison between this run and run (e) showed that peak (4) is myricetin which was superimposed above carmine. By comparing run (a), (e) and (f), it was concluded that the last peak in run (a) and (f) belonged to apigenin.

Figure 3.17-g: run (a) without carmine (4) and morin (5)

In this run carmine and morin were excluded from the mixture of dyes. Regarding carmine the data from run (d), (e), (f) established that in run (a) carmine peak was under the peak

was under the peak of myricetin. From run (b) it was concluded that quercetin was superimposed above morin.

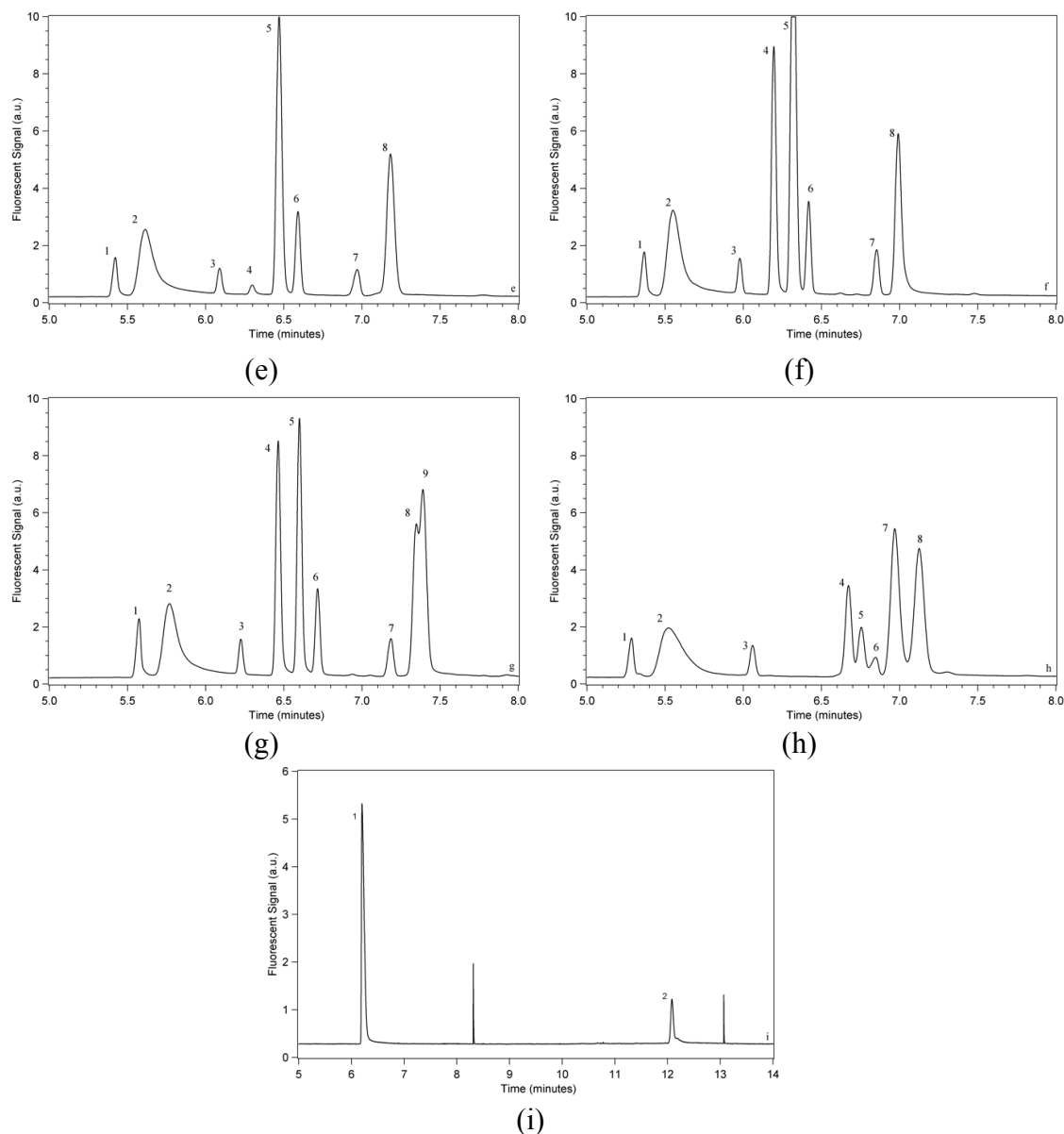


Figure 3.17 continued Peak identities: run (e): (1) alizarin, (2) purpurin, (3) emodin, (4) carmine, (5) quercetin and morin, (6) luteolin, (7) the impurity from emodin, (8) apigenin. run (f): (1) alizarin, (2) purpurin, (3) emodin, (4) myricetin, (5) quercetin and morin, (6) luteolin, (7) the impurity from emodin, (8) apigenin. run (g): (1) alizarin, (2) purpurin, (3) emodin, (4) myricetin (5) quercetin, (6) luteolin, (7) the impurity from emodin, (8) kaempferol, and (9) apigenin. run (h): (1) alizarin, (2) purpurin, (3) emodin, (4) morin, (5) luteolin, (6) the impurity from emodin, (7) kaempferol, and (8) apigenin. Run (i): (1) emodin, (2) the impurity from emodin.

Figure 3.17-h: run (a) without carmine (4), myricetin (4) and quercetin (5)

In this run, there was no peak between emodin (peak 3) and morin (peak 4), and it can be used as another proof of overlapping myricetin and carmine. This finding was in agreement with results from runs (d), (e), (f) and (g). In this run by removing quercetin, the peak belonging to morin was revealed and established the conclusions made in run (b) and (g).

Figure 3.17-i: only emodin

Run (i) consisted only of emodin and revealed 2 peaks showing that peak (1) and (2) belonged to emodin and contamination, respectively. Peak 2 in this run is consistent with peak (7) in runs (a), (b), (e), and (f).

From this study it was concluded that at pH= 8.0 the baseline separation for most dyes was achieved except in the case of carmine superimposed by myricetin and morin superimposed by quercetin. Different amounts of acetonitrile and methanol, as well as different applied voltages could not resolve these peaks. Therefore, the effect of higher pH was explored. It is worth mentioning that the separation of selected flavonoids using MEKC: 50 mM phosphate with 25 mM SDS and 25 mM sodium cholate (trihydroxy bile salt) at pH= 7.0, $l = 40$ cm and $V = 20$ kV was in agreement with our findings where kaempferol and apigenin eluted last after morin and quercetin [50]. The elution order of selected flavonoids in food sources using MEKC: 25 mM borate with 40 mM SDS at pH~8.2 and $V = 20$ kV showed the following elution order: luteolin, quercetin and apigenin, where the first 2 compounds eluted at 7:40 and 7:53 minute, and apigenin eluted at 11:30 minute, which is similar to our observation at pH=8.0 [51].

3.4.5 The effect of Modifier on separation at pH= 8.5

From **Figures 3.11, 3.12 and 3.13** it was concluded that the baseline separation of the mixture of selected dyes was not achieved at pH= 8.0. Since, using modifiers and different voltages could not resolve the peaks, it was decided to explore the separation at pH= 8.5.

The separation of a mixture of anthraquinones and flavonoids including 9 dyes at pH= 8.5 was investigated. It was noticed that instead of 9 peaks only 7 peaks appeared. Therefore, 2 dyes should have overlapped (**Figure 3.18-a**). In order to separate these dyes, 5, 10 and 20% (v/v) of acetonitrile or methanol was used; the results are given in **Figures 3.18 and 3.19**. The Application of 20% modifiers did not help to resolve the peaks and therefore this data is not presented. In order to have a better view of peak shapes the close up of each run is also included.

In the next section, the identification of dyes is presented and by using this information, the peaks in **Figure 3.18-a** are assigned as: (1) alizarin, (2) emodin, (3) purpurin, (4) Kaempferol, (5) apigenin/myricetin, (6) luteolin/quercetin and (7) morin.

Cao *et al.* studied the migration time of quercetin and luteolin as a function of pH using 100 mM borate, V= 30 kV and it was shown that these 2 dyes had the same migration time at pH range 8.4- 8.8. Therefore, the reason for these 2 dyes overlapping can be due to having the same mobility [52].

Application of 5% acetonitrile resolved peaks of myricetin (5) and apigenin (6) as well as luteolin (7) and quercetin (8) (**Figure 3.18-b**). Therefore, in the presence of 5% acetonitrile at pH= 8.5 the baseline separation of 9 dyes was achieved.

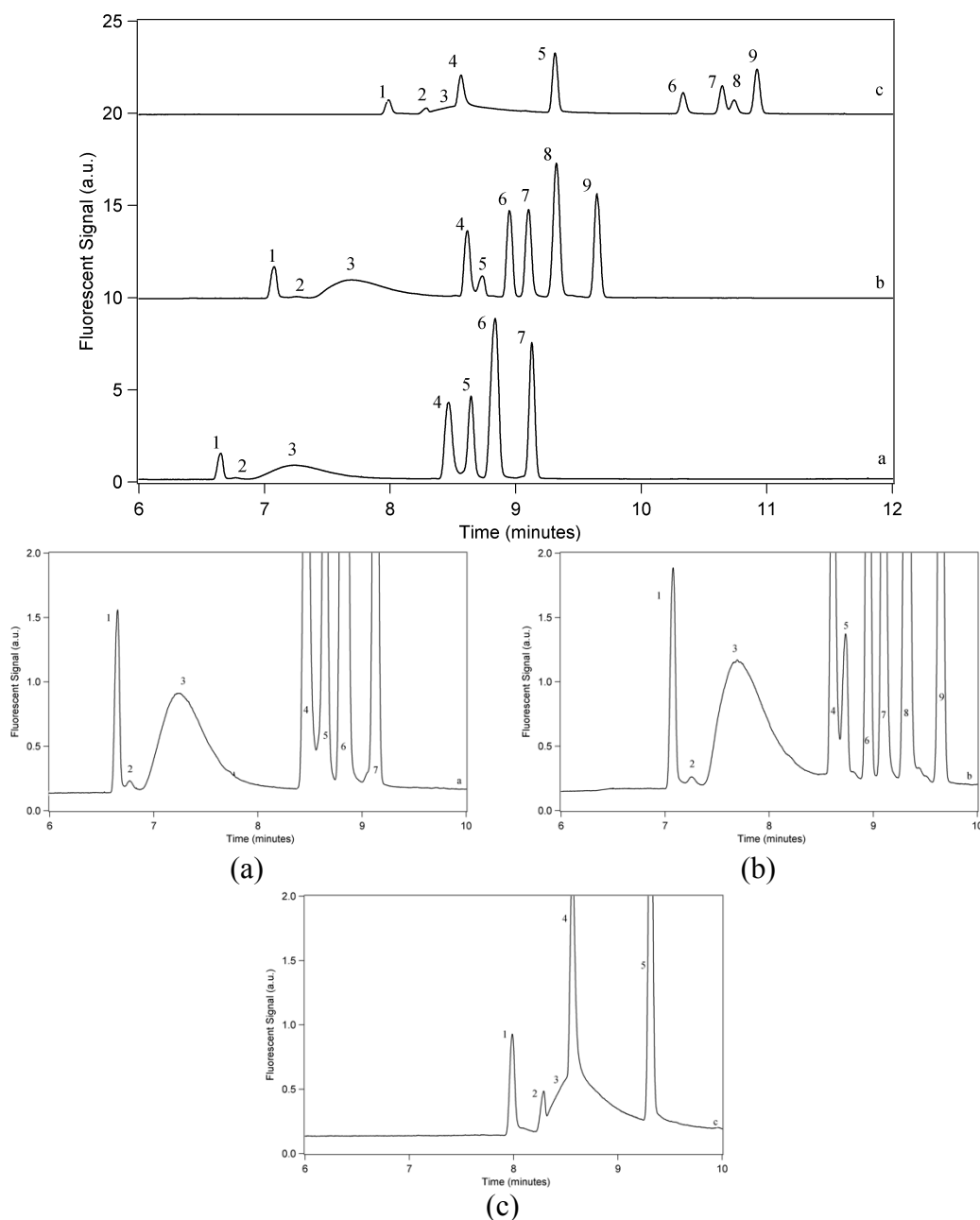


Figure 3.18 Separation of selected anthraquinones and flavonoid dyes ($1 \mu\text{g ml}^{-1}$ alizarin, $3 \mu\text{g ml}^{-1}$ purpurin, $1 \mu\text{g ml}^{-1}$ emodin, $2 \mu\text{g ml}^{-1}$ myricetin, $1 \mu\text{g ml}^{-1}$ quercetin, $0.5 \mu\text{g ml}^{-1}$ morin, $2 \mu\text{g ml}^{-1}$ luteolin, $0.5 \mu\text{g ml}^{-1}$ kaempferol, $1 \mu\text{g ml}^{-1}$ apigenin,) in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, (a) no modifier, (b) in 5% (v/v), and (c) in 10% (v/v) of acetonitrile at 18 kV. The assigned peaks for run **(a)**: (1) alizarin, (2) emodin, (3) purpurin, (4) Kaempferol, (5) apigenin/ myricetin, (6) luteolin/ quercetin and (7) morin. Run **(b)**: (1) alizarin, (2) emodin, (3) purpurin, (4) Kaempferol, (5) myricetin, (6) apigenin, (7) luteolin, (8) quercetin and (9) morin. Run **(c)**: (1) alizarin, (2) emodin, (3) purpurin, (4) Kaempferol, (5) apigenin, (6) myricetin, (7) luteolin, (8) quercetin and (9) morin.

In the presence of 10 % acetonitrile the dyes separated with a poor quality and low fluorescence signal, especially in the case of kaempferol (4), which eluted with purpurin (3).

The effect of methanol as a modifier was explored to separate 10 dyes (the same mixture used in Figure 3.18 including carmine) and the results are presented in **Figure 3.19**. In **Figure 3.19-a**, instead of 10 peaks, only 7 peaks were detected. By using 5% and 10% methanol the baseline separation of dyes was investigated. Using 5% methanol in run (b) did not allow the resolution of the peaks and there were overlaps between kaempferol and emodin (3), apigenin, carmine and myricetin (4), and luteolin and quercetin (5).

By using 10% methanol the baseline separation of most dyes was achieved except for purpurin (2), kaempferol (3) and emodin (4). The elution order was defined as: alizarin (1), purpurin (2), kaempferol (3) and emodin (4), apigenin (5), carmine (6), myricetin (7), luteolin (8), quercetin (9), and morin (10).

For the separation of flavonoids in honey Delgado *et al.* [53] used MEKC: 200 mM boric acid, 50 mM SDS at pH= 8.5 and V= 20 kV, and found the following order between 12 to 19 minutes: myricetin, quercetin and luteolin, kaempferol, and apigenin. This order is in agreement with our findings at pH= 8.0. But the findings reported by Delgado *et al.* at pH= 8.5 with 150 mM boric acid and 75 mM SDS resulted in separation of quercetin and luteolin although luteolin eluted prior to myricetin. It shows how the elution order can be affected by concentration of buffer and SDS [53]. Ng *et al.* used 50 mM borate/ phosphate buffer and 30 mM SDS at pH= 8.5 found that morin eluted prior to quercetin [54].

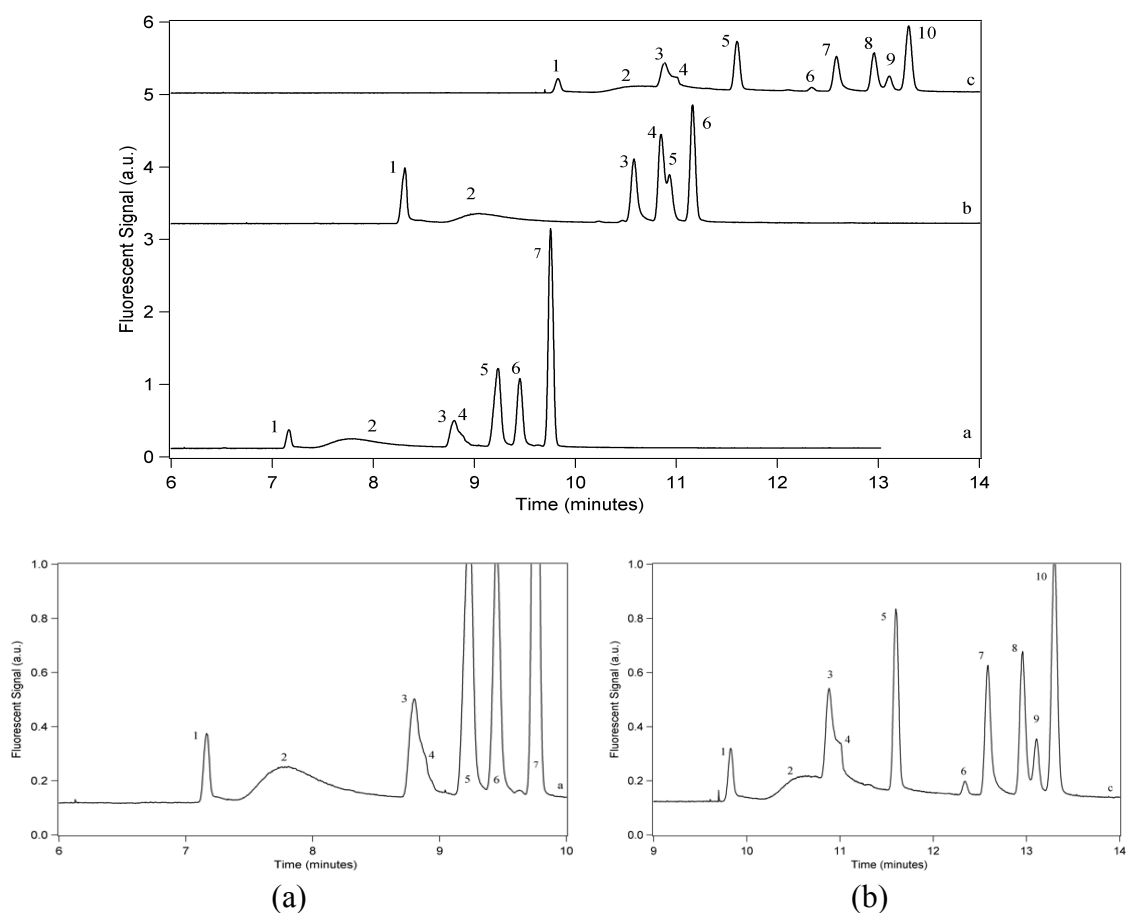


Figure 3.19 Separation of selected anthraquinones and flavonoid dyes ($3 \mu\text{g ml}^{-1}$ alizarin, $3 \mu\text{g ml}^{-1}$ purpurin, $1 \mu\text{g ml}^{-1}$ emodin, $2 \mu\text{g ml}^{-1}$ myricetin, $10 \mu\text{g ml}^{-1}$ carmine, $1 \mu\text{g ml}^{-1}$ quercetin, $0.5 \mu\text{g ml}^{-1}$ morin, $2 \mu\text{g ml}^{-1}$ luteolin, $0.5 \mu\text{g ml}^{-1}$ kaempferol, $1 \mu\text{g ml}^{-1}$ apigenin,) in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, run **(a)** no modifier, **(b)** in 5% (v/v), and **(c)** in 10% (v/v) of methanol at 18 kV. The assigned peaks for run **(a)**: (1) alizarin, (2) purpurin, (3) Kaempferol, (4) emodin, (5) apigenin/ myricetin/ carmine, (6) luteolin/ quercetin and (7) morin. Run **(b)**: (1) alizarin, (2) purpurin, (3) Kaempferol/ emodin, (4) apigenin/ myricetin/carmine, (5) luteolin/ quercetin and (6) morin. Run **(c)**: (1) alizarin, (2) purpurin, (3) Kaempferol, (4) emodin, (5) apigenin, (6) carmine, (7) myricetin, (8) luteolin, (9) quercetin and (10) morin.

3.4.6 The effect of voltage on separation at pH= 8.5

The effect of applied voltage (18-25 kV) on the separation of these dyes with and without modifier was explored. The results for separation of dyes without modifier are shown in

Figure 3.20. Figure 3.21 and 3.22 show the effect of voltage on separation in the presence of 5% acetonitrile and methanol, respectively.

From **Figure 3.20** it was concluded that apigenin eluted at different elution times; in run (a) and (d) after and in run (b) before myricetin. The best baseline separation for most dyes especially carmine (6) was achieved at 20 kV (run b). In this run emodin (4) eluted as a shoulder peak of kaempferol (3). Therefore, in following section, the modifiers were used to resolve peaks.

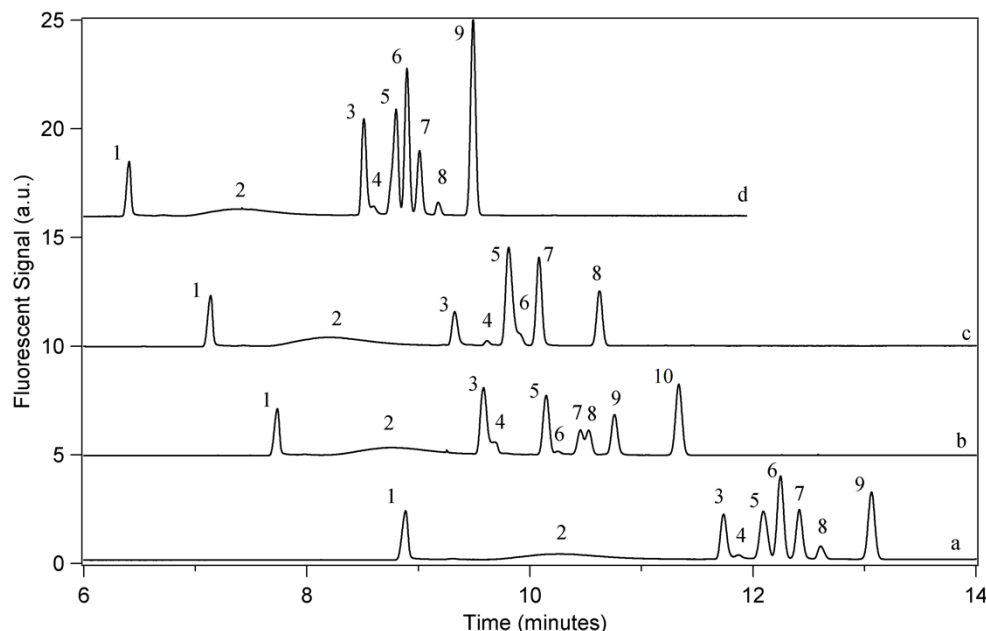


Figure 3.20 Separation of selected anthraquinones and flavonoid dyes (the same concentration used in Figure 3.19) in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, at: **(a)** 18 kV, **(b)** 20 kV, **(c)** 22 kV and **(d)** 25 kV. The assigned peaks for **(a)**: (1) alizarin, (2) purpurin, (3) kaempferol, (4) emodin, (5) myricetin, (6) apigenin/ carmine, (7) luteolin, (8) quercetin, (9) morin. Run **(b)**: (1) alizarin, (2) purpurin, (3) kaempferol, (4) emodin, (5) apigenin, (6) carmine, (7) myricetin, (8) luteolin, (9) quercetin, and (10) morin. Run **(c)**: (1) alizarin, (2) purpurin, (3) kaempferol, (4) emodin, (5) apigenin/ myricetin, (6) carmine, (7) luteolin/ quercetin, (8) morin. Run **(d)**: (1) alizarin, (2) purpurin, (3) kaempferol, (4) emodin, (5) myricetin, (6) apigenin/ carmine, (7) luteolin (8) quercetin, and (9) morin.

Figure 3.21 presents the results of separation of dyes at different voltages using 5% (v/v) acetonitrile. It was concluded using 5% acetonitrile resolved carmine (6) and the best

baseline separation considering peak shapes and elution time was achieved at 22 kV. It was noticed that at lower voltages 18 kV and 20 kV (run a and b) emodin (3) eluted before kaempferol (4) and by increasing the voltage the order reversed. Except for these 2 dyes, the elution order for all runs was assigned as: alizarin (1), purpurin (2) emodin, kaempferol (3,4), apigenin (5), carmine (6), myricetin (7), luteolin (8), quercetin (9) and morin (10).

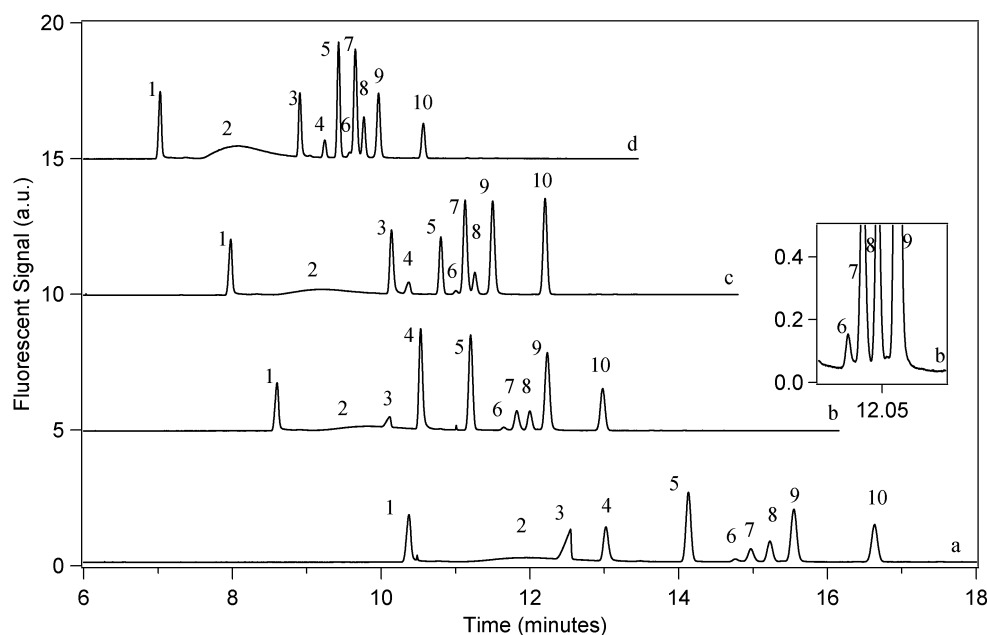


Figure 3.21 Separation of selected anthraquinones and flavonoid dyes (as mentioned in Figure 3.19) in presence 5% (v/v) of acetonitrile in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, at: **(a)** 18 kV, **(b)** 20 kV **(c)** 22 kV and **(d)** 25 kV. The assigned peaks for **(a)** and **(b)** : (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin, (6) carmine, (7) myricetin, (8) luteolin, (9) quercetin, (10) morin. Run **(c)** and **(d)**: (1) alizarin, (2) purpurin, (3) kaempferol, (4) emodin, the rest is the same as in run (a).

The effect of 5% (v/v) methanol was explored and the results are shown in **Figure 3.22**. The comparison of the peaks showed that using 18 kV run (a), emodin (4) eluted as a shoulder peak of apigenin (5) and it superimposed carmine. It can be concluded that the baseline separation of dyes was achieved using 20 kV and 22 kV (runs b and c), except for emodin (3), which eluted at the same time with purpurin (2). As mentioned before

Puchalska *et al.* reported the co-elution of purpurin and emodin using MEKC at pH= 8.0, 8.5 and 9.0 [19].

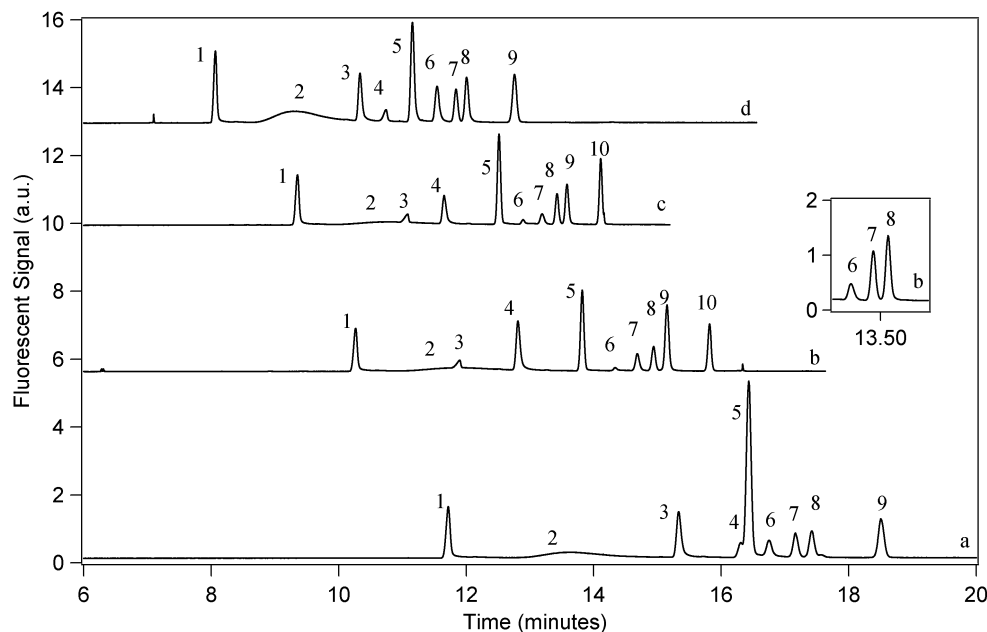


Figure 3.22 Separation of selected anthraquinones and flavonoid dyes (as mentioned in Figure 3.19) in presence 5% (v/v) of methanol in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, at: **(a)** 18 kV, **(b)** 20 kV **(c)** 22 kV and **(d)** 25 kV. The assigned peaks for run **(a)** and **(d)**: (1) alizarin, (2) purpurin, (3) kaempferol, (4) emodin, (5) apigenin/carmine (6) myricetin, (7) luteolin, (8) quercetin, (9) morin. Run **(b)** and **(c)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin, (6) carmine, (7) myricetin, (8) luteolin, (9) quercetin, (10) morin.

3.4.7 Identification of dyes at pH= 8.5

In order to assign the peaks at pH= 8.5 a series of runs were performed. The first run consisted of a known mixture while in following runs dyes were added one at a time and the peaks were compared; the results are summarized in **Figure 3.23**.

Figure 3.23-a shows the separation of a mixture of dyes. The eluted peaks were assigned as alizarin (1), purpurin (2), carmine (3) and morin (4). A fresh solution used in run (a) was prepared for run (b) including emodin. The comparison between these 2 runs showed that the new peak (3) located between purpurin and carmine belonged to emodin.

Myricetin was added in run (c) and comparison of the results confirmed that peak (5) belonged to myricetin. Quercetin was added to the mixture in run (d) and its peak did not show up. The comparison between peak (5) in run (c) and (d), shows that the height of this peak had increased and it was evident that quercetin should have overlapped with myricetin.

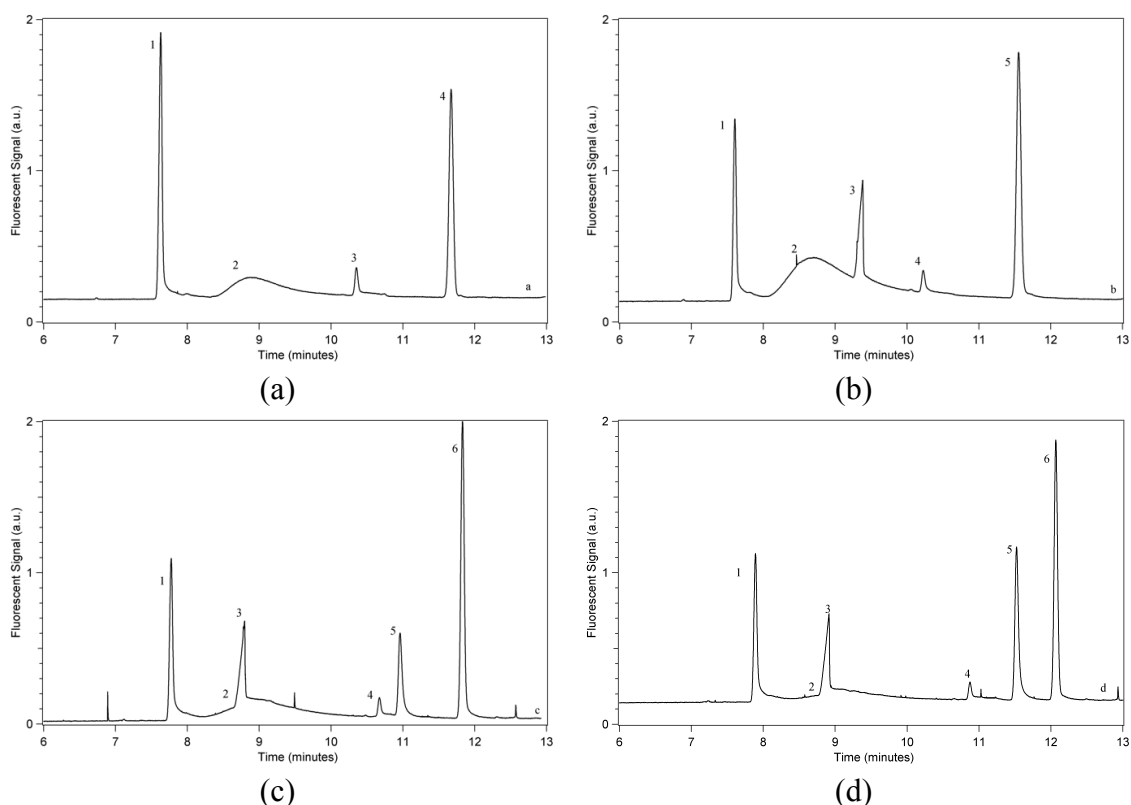


Figure 3.23 Separation of a mixture of dyes in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, in 5% (v/v) of methanol, using an applied potential of 25 kV. The concentrations of dyes were: $3 \mu\text{g ml}^{-1}$ alizarin, $3 \mu\text{g ml}^{-1}$ purpurin, $5 \mu\text{g ml}^{-1}$ emodin, $2 \mu\text{g ml}^{-1}$ myricetin, $10 \mu\text{g ml}^{-1}$ carmine, $2 \mu\text{g ml}^{-1}$ quercetin, $0.2 \mu\text{g ml}^{-1}$ morin. Peak identities: run (a): (1) alizarin, (2) purpurin, (3) carmine, (4) morin. Run (b): (1) alizarin, (2) purpurin, (3) emodin, (4) carmine, (5) morin. Run (c): (1) alizarin, (2) purpurin, (3) emodin, (4) carmine, (5) myricetin, (6) morin. Run (d): (1) alizarin, (2) purpurin, (3) emodin, (4) carmine, (5) myricetin/ quercetin, (6) morin.

From this study the following order was concluded: alizarin (1), purpurin (2), emodin (3), carmine (4), myricetin (5), quercetin (6) and morin (7).

In order to identify dyes the separation was performed without modifier at 18 kV. The results are presented in **Figure 3.24**. In the first run alizarin (1), purpurin (2), and morin (3) were injected and the result of this run was compared with run (b), where emodin was added to the mixture and it was assumed that the fourth peak belonged to emodin. The concentration of emodin was increased in run (c). Peak (3) is the only one that increased in size, so it was concluded that by increasing the amount of emodin, its elution order could change. It eluted earlier than morin as a shoulder peak.

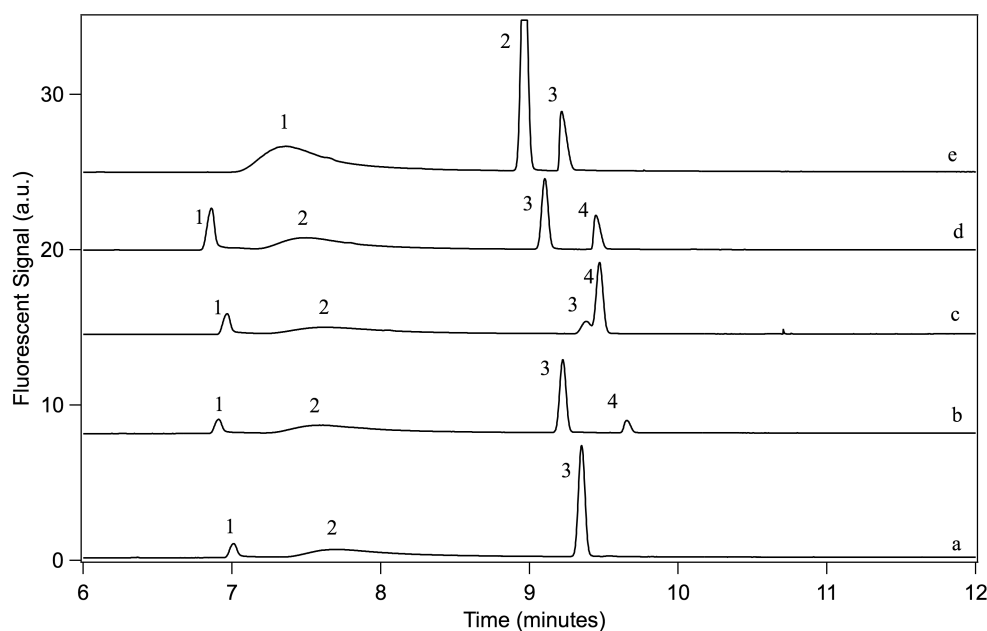


Figure 3.24 Separation of a mixture of dyes in a 20 mM borate buffer and 20 mM SDS at pH= 8.5 using an applied potential of 18 kV. Peak identities: run **(a)**: (1) $3 \mu\text{g ml}^{-1}$ alizarin, (2) $3 \mu\text{g ml}^{-1}$ purpurin, (3) $0.5 \mu\text{g ml}^{-1}$ morin. Run **(b)**: (1) $3 \mu\text{g ml}^{-1}$ alizarin, (2) $3 \mu\text{g ml}^{-1}$ purpurin, (3) $0.5 \mu\text{g ml}^{-1}$ morin, (4) $1 \mu\text{g ml}^{-1}$ emodin, (5). Run **(c)**: (1) $5 \mu\text{g ml}^{-1}$ alizarin, (2) $3 \mu\text{g ml}^{-1}$ purpurin, (3) $5 \mu\text{g ml}^{-1}$ emodin, (5) $0.5 \mu\text{g ml}^{-1}$ morin. Run **(d)**: (1) $10 \mu\text{g ml}^{-1}$ alizarin, (2) $3 \mu\text{g ml}^{-1}$ purpurin, (3) $0.5 \mu\text{g ml}^{-1}$ morin, (4) $5 \mu\text{g ml}^{-1}$ emodin. Run **(e)**: (1) $5 \mu\text{g ml}^{-1}$ alizarin and $10 \mu\text{g ml}^{-1}$ purpurin, (2) $\mu\text{g ml}^{-1}$ morin, (3) $10 \mu\text{g ml}^{-1}$ emodin.

Figure 3.24-d shows the separation of a fresh solution of 4 dyes and emodin eluting after morin. In the last run, increasing the concentration of purpurin and morin caused an overlap peak of alizarin and purpurin (1), and off-scale peak for morin (2), respectively.

The elution order of dyes under this condition should be as follow: alizarin, purpurin, morin, emodin (or emodin, morin). This order is different from the order concluded from the combination of elution time of each class of dyes where morin eluted last and emodin was between purpurin and morin.

Figure 3.25 shows the elution of a mixture consisting of, myricetin (b), quercetin (c), apigenin (d), emodin (e), kaempferol (f), and luteolin (g).

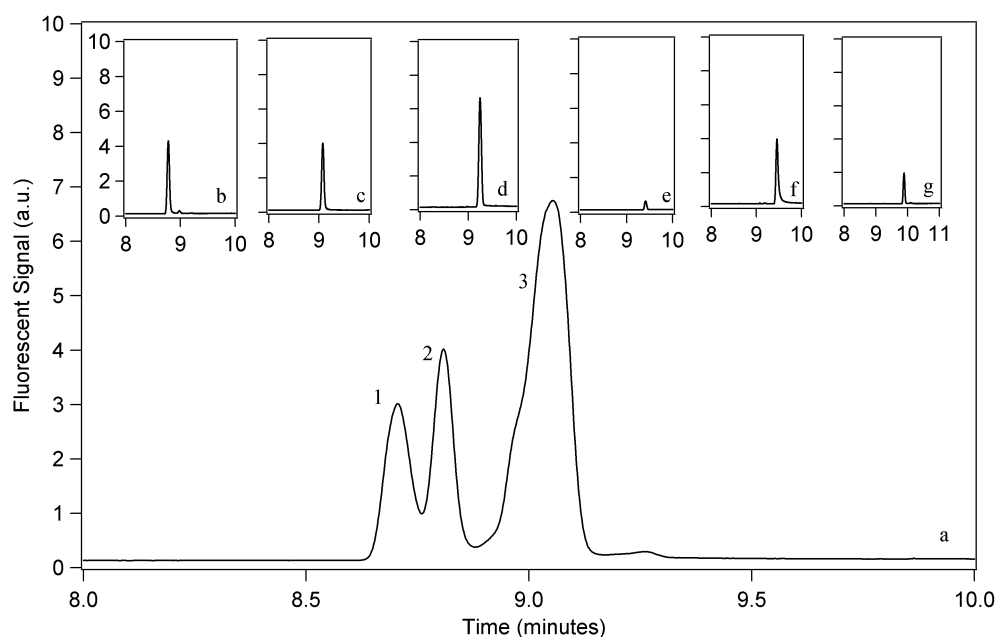


Figure 3.25 Separation of a mixture of dyes in a 20 mM borate buffer and 20 mM SDS at pH= 8.5 using an applied potential of 18 kV. Peak identities: Run (a): (1) $0.5 \mu\text{g ml}^{-1}$ kaempferol and $1 \mu\text{g ml}^{-1}$ emodin, (2) $1 \mu\text{g ml}^{-1}$ apigenin and (3) $2 \mu\text{g ml}^{-1}$ myricetin, $1 \mu\text{g ml}^{-1}$ quercetin and $2 \mu\text{g ml}^{-1}$ luteolin. (b): $2 \mu\text{g ml}^{-1}$ myricetin, (c): $1 \mu\text{g ml}^{-1}$ quercetin, (d): $1 \mu\text{g ml}^{-1}$ apigenin, (e): $1 \mu\text{g ml}^{-1}$ emodin, (f): $0.5 \mu\text{g ml}^{-1}$ kaempferol, and (g): $2 \mu\text{g ml}^{-1}$ luteolin.

In this run instead of 6 peaks, only 3 peaks appeared because some dyes overlapped with each other. In order to assign these peaks each dye was injected individually and its elution time was compared with the peaks in run (a). It can be concluded that: peak (1) is the overlapping of emodin (e) and kaempferol (f), peak (2) belonged to apigenin (d), and peak (3) was the overlapping of myricetin (b), quercetin (c), and luteolin (g). This finding is in agreement with the order concluded for separation of each class of dyes (emodin, kaempferol /apigenin, myricetin, luteolin, quercetin and morin).

Figure 3.25-a shows the elution of a mixture consisting of, myricetin (b), quercetin (c), apigenin (d), emodin (e), kaempferol (f), and luteolin (g). In this run, instead of 6 peaks, only 3 peaks appeared because some dyes overlapped with each other. In order to assign these peaks each dye was injected individually and its elution time was compared with the peaks in run (a). It can be concluded that: peak (1) is the overlapping of emodin (e) and kaempferol (f), peak (2) belonged to apigenin (d), and peak (3) was the overlapping of myricetin (b), quercetin (c), and luteolin (g). This finding is in agreement with the order concluded for separation of each class of dyes (emodin, kaempferol/apigenin, myricetin, luteolin, quercetin and morin).

In **Figure 3.26** a mixture of 8 flavonoids were injected and it was noticed that there was an overlap between 2 peaks. The same dyes were injected in run (b) and concentration of apigenin (4) and quercetin (6) were increased; kaempferol (3) and luteolin (5) were decreased. The comparison of changes in the sizes of peaks helped to assign them.

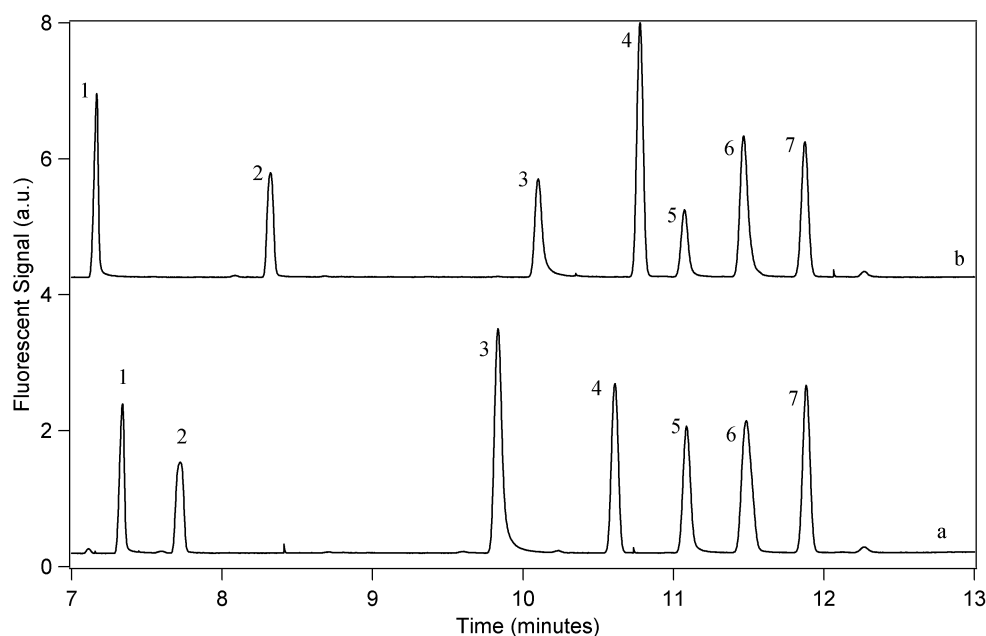


Figure 3.26 Separation of a mixture of flavonoids in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, in 5% (v/v) of acetonitrile, using an applied potential of 22 kV. Peak identities: Run **(a)**: (1) $2 \mu\text{g ml}^{-1}$ lu-7-*O*-glu, (2) $2 \mu\text{g ml}^{-1}$ ap-7-*O*-glu, (3) $2 \mu\text{g ml}^{-1}$ kaempferol, (4) $2 \mu\text{g ml}^{-1}$ apigenin, $6 \mu\text{g ml}^{-1}$ myricetin, (5) $2 \mu\text{g ml}^{-1}$ luteolin, (6) $1 \mu\text{g ml}^{-1}$ quercetin, (7) $1 \mu\text{g ml}^{-1}$ morin. Run **(b)**: (1) $2 \mu\text{g ml}^{-1}$ lu-7-*O*-glu, (2) $2 \mu\text{g ml}^{-1}$ ap-7-*O*-glu, (3) $1 \mu\text{g ml}^{-1}$ kaempferol, (4) $4 \mu\text{g ml}^{-1}$ apigenin, $6 \mu\text{g ml}^{-1}$ myricetin, (4) $1 \mu\text{g ml}^{-1}$ luteolin, (6) $2 \mu\text{g ml}^{-1}$ quercetin, (7) $1 \mu\text{g ml}^{-1}$ morin.

It was hypothesized that myricetin and apigenin (4) eluted at the same time. In this study the elution order of flavonoids at pH= 8.5 was assigned as: lu-7-*O*-glu, ap-7-*O*-glu, kaempferol, apigenin/myricetin, luteolin, quercetin, morin.

In **Figure 3.27** the mixture of flavonoids excluding myricetin was injected and it proved that peak (4) was an overlap of myricetin and apigenin. In this run peak (5) was the overlap of luteolin and quercetin. From Figure 3.27 the following order was concluded: lu-7-*O*-glu, ap-7-*O*-glu, kaempferol, apigenin, luteolin/quercetin, and morin.

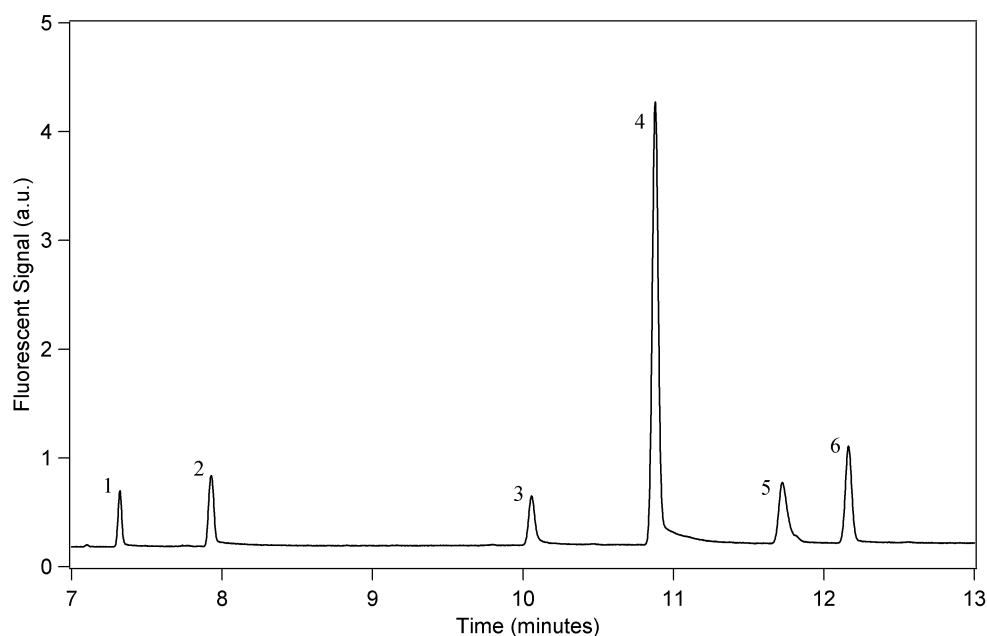


Figure 3.27 Separation of a mixture of flavonoids in a 20 mM borate buffer and 20 mM SDS at pH= 8.5, in 5% (v/v) of acetonitrile, using an applied potential of 22 kV. Peak identities: (1) $2 \mu\text{g ml}^{-1}$ lu-7-*O*-glu, (2) $2 \mu\text{g ml}^{-1}$ ap-7-*O*-glu, (3) $1 \mu\text{g ml}^{-1}$ kaempferol, (4) $1 \mu\text{g ml}^{-1}$ apigenin, (5) $1 \mu\text{g ml}^{-1}$ luteolin/ $1 \mu\text{g ml}^{-1}$ quercetin, (6) $1 \mu\text{g ml}^{-1}$ morin.

3.4.8 The effect of Modifier on separation at pH= 9.2

The separation of anthraquinones and flavonoids were investigated at pH= 9.2. **Figure 3.28-a** shows the separation of a mixture of 10 dyes without modifier. It was noticed that there should be an overlap between 2 peaks since only 9 peaks showed up. The results from **Figure 3.28** showed that apigenin and luteolin overlapped.

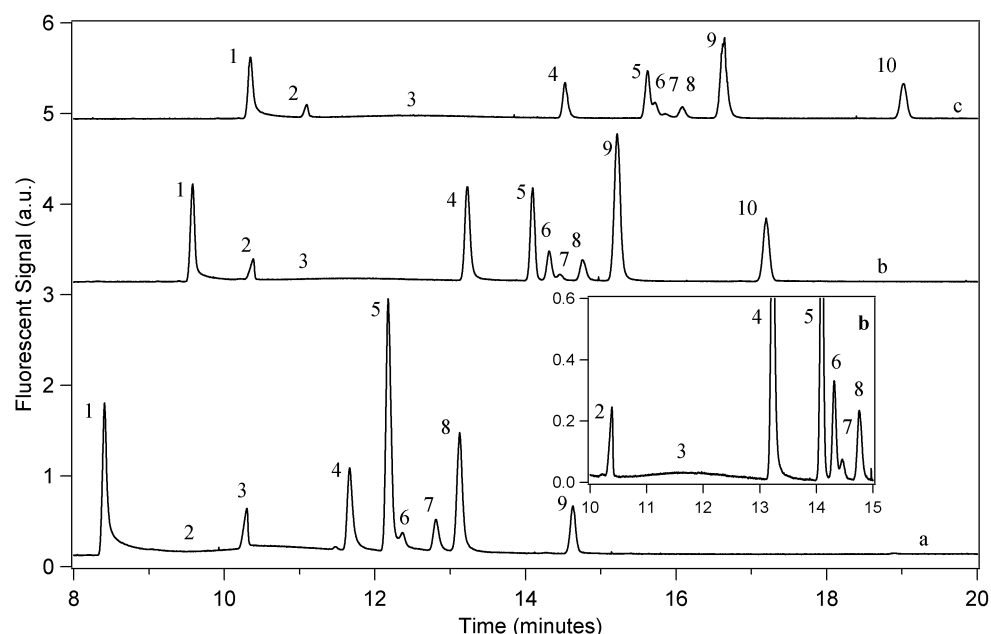


Figure 3.28 Separation of selected anthraquinones and flavonoid dyes ($5 \mu\text{g ml}^{-1}$ alizarin, $10 \mu\text{g ml}^{-1}$ purpurin, $1 \mu\text{g ml}^{-1}$ emodin, $0.5 \mu\text{g ml}^{-1}$ kaempferol, $1 \mu\text{g ml}^{-1}$ apigenin, $2 \mu\text{g ml}^{-1}$ luteolin, $10 \mu\text{g ml}^{-1}$ carmine, $2 \mu\text{g ml}^{-1}$ myricetin, $1 \mu\text{g ml}^{-1}$ quercetin, $0.5 \mu\text{g ml}^{-1}$ morin), in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, **(a)** no modifier, **(b)** in 5% (v/v), and **(c)** in 10% (v/v) of acetonitrile at 20 kV. The assigned peaks for **(a)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin/luteolin, (6) carmine, (7) myricetin, (8) quercetin and (9) morin. Run **(b)** and **(c)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin, (6) luteolin, (7) carmine, (8) myricetin, (9) quercetin and (10) morin.

By using the data from **Figure 3.35– Figure 3.40** (next section) the peaks were assigned as: alizarin (1), emodin (2), purpurin (3), kaempferol (4), apigenin/ luteolin (5), carmine (6), myricetin (7), quercetin (8) and morin (9). Experiments were carried out to examine the effect of organic modifier concentration on the separation of these dyes using 5-20% methanol or acetonitrile. Figure 3.28-b shows that using 5% acetonitrile could resolve apigein (5) and luteolin (6). Increasing the amount of acetonitrile resulted in co elution of apigenin (5) and luteolin (6). Moreover, 10% ACN did not improve the baseline separation of carmine (7). **Figure 3.29** shows a close up of run b and c.

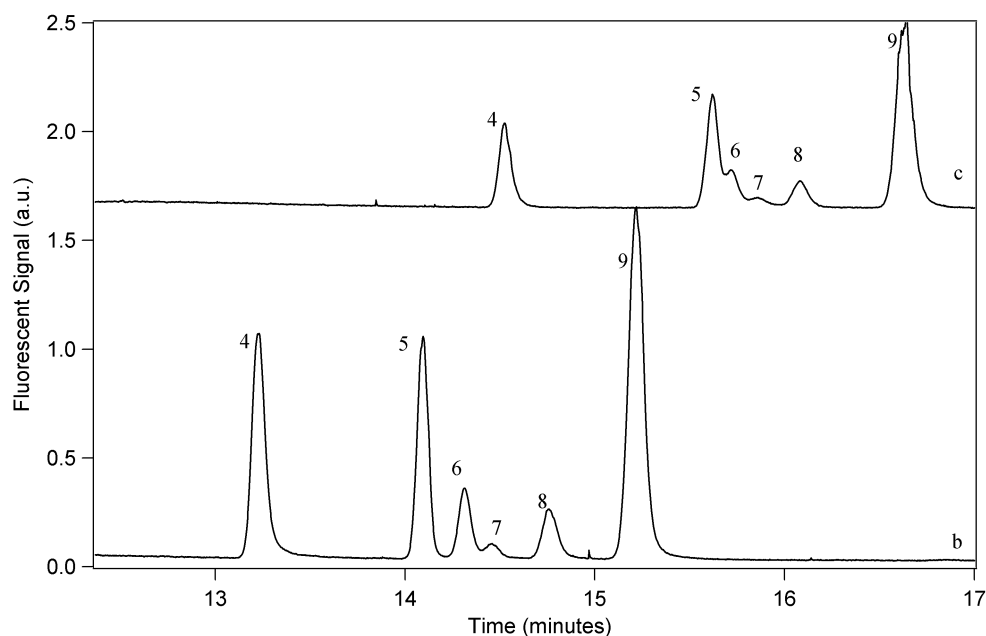


Figure 3.29 A close up of run b and c in Figure 3.28

The elution order of flavonoids was consistent with data published by others groups using similar separation conditions (e.g. ionic strength) [48, 54].

The effect of methanol on separation of the mixture of dyes was investigated and the results are presented in Figure 3.30 and 3.31. In **Figure 3.30-a** a mixture of 9 dyes was injected and the number of peaks indicated that there was an overlap between 2 dyes. The peaks were assigned as: alizarin (1), emodin (2), purpurin (3), kaempferol (4), apigenin/luteolin (5), myricetin (6), quercetin (7) and morin (8). The comparison of run (b) and (c) indicated that the baseline separation of 9 dyes was achieved in the presence of 5% methanol and it could separate luteolin from apigenin. However, increasing the amount of methanol did not resolve the peaks and it caused the co-elution of luteolin and myricetin (run c, peak 6).

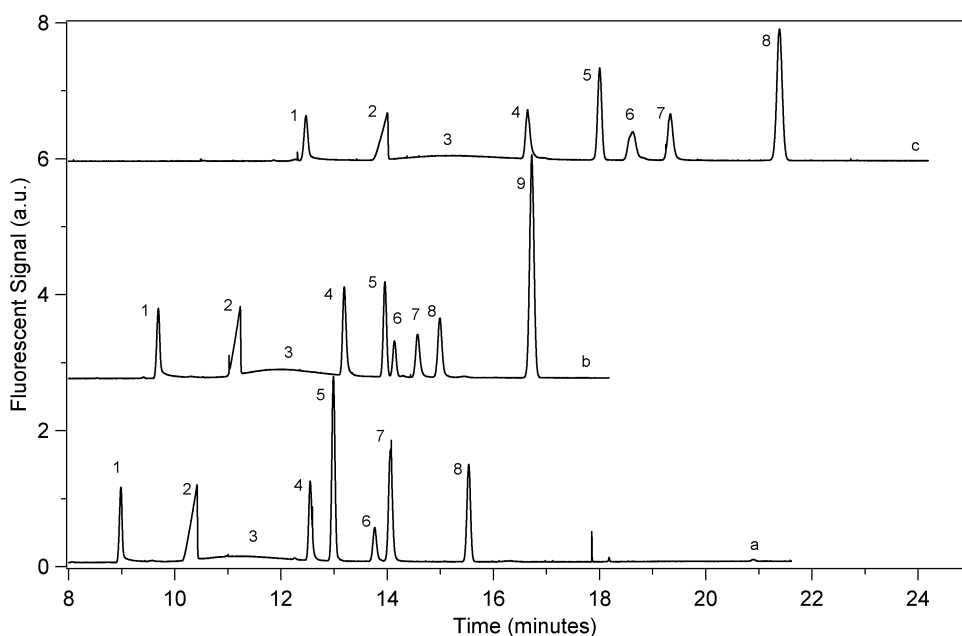


Figure 3.30 Separation of selected anthraquinones and flavonoid dyes ($5 \mu\text{g ml}^{-1}$ alizarin, $10 \mu\text{g ml}^{-1}$ purpurin, $10 \mu\text{g ml}^{-1}$ emodin, $0.5 \mu\text{g ml}^{-1}$ kaempferol, $1 \mu\text{g ml}^{-1}$ apigenin, $2 \mu\text{g ml}^{-1}$ luteolin, $2 \mu\text{g ml}^{-1}$ myricetin, $1 \mu\text{g ml}^{-1}$ quercetin, $0.5 \mu\text{g ml}^{-1}$ morin), in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, **(a)** no modifier, **(b)** in 5% (v/v), and **(c)** in 10% (v/v) of methanol at 20 kV. The assigned peaks for **(a)**: (1) alizarin, (2) emodin, (3) purpurin, (4) kaempferol, (5) apigenin/luteolin, (6) myricetin, (7) quercetin and (8) morin. Run **(b)**: (1) alizarin, (2) emodin, (3) purpurin, (4) kaempferol, (5) apigenin, (6) luteolin, (7) myricetin, (8) quercetin and (9) morin; run **(c)** (1) alizarin, (2) emodin, (3) purpurin, (4) kaempferol, (5) apigenin, (6) luteolin/ myricetin, (7) quercetin and (8) morin.

Figure 3.31 shows the effect of methanol on separation of a mixture used in Figure 3.30 including carmine. The separation of ten dyes are presented in **Figure 3.31-a**. The number of peaks in this run indicated that there was an overlap between 2 dyes. From the findings of Figure 3.30 it was concluded that peak (5) is the overlap of apigenin and luteolin (5); it was also noticed that carmine (6) was eluted as a shoulder peak of apigenin. By using 5% methanol in run (b) luteolin (5) resolved from apigenin (6) but the baseline separation of carmine did not take place. It seems apigenin (6) superimposed carmine. Application of

10% of methanol in run (c) did not help the baseline separation of luteolin and apigenin, nor carmine.

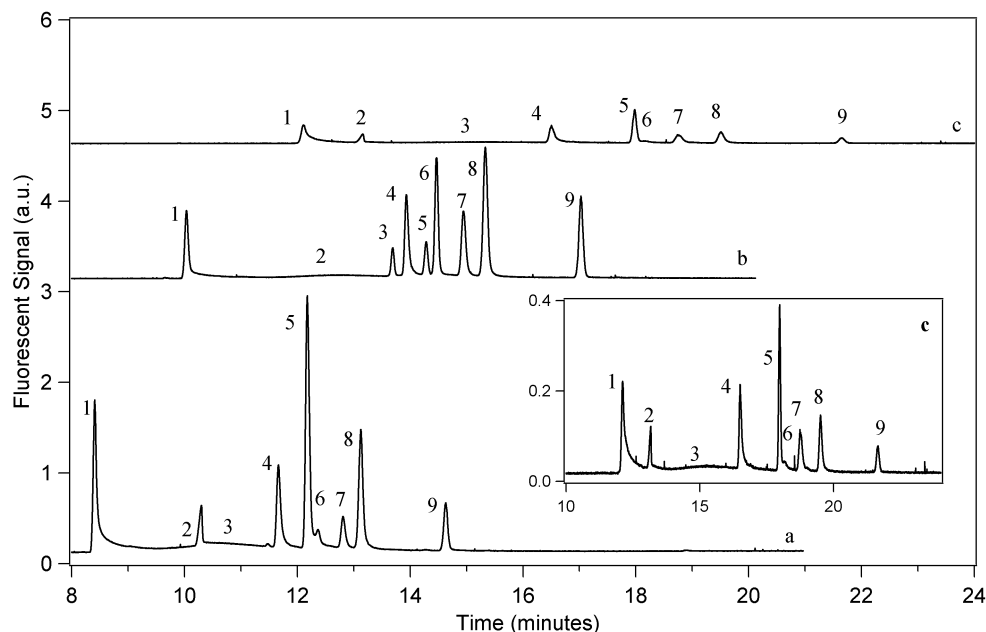


Figure 3.31 Separation of selected anthraquinones and flavonoid dyes ($10 \mu\text{g ml}^{-1}$ alizarin, $10 \mu\text{g ml}^{-1}$ purpurin, $1 \mu\text{g ml}^{-1}$ emodin, $0.5 \mu\text{g ml}^{-1}$ kaempferol, $1 \mu\text{g ml}^{-1}$ apigenin, $2 \mu\text{g ml}^{-1}$ luteolin, $10 \mu\text{g ml}^{-1}$ carmine, $2 \mu\text{g ml}^{-1}$ myricetin, $1 \mu\text{g ml}^{-1}$ quercetin, $0.5 \mu\text{g ml}^{-1}$ morin), in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, **(a)** no modifier, **(b)** in 5% (v/v), and **(c)** in 10% (v/v) of methanol at 20 kV. The assigned peaks for **(a)** and **(c)**: (1) alizarin, (2) emodin, (3) purpurin, (4) kaempferol, (5) apigenin/luteolin, (6) carmine, (7) myricetin, (8) quercetin and (9) morin. Run **(b)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) luteolin, (6) apigenin/carmine, (7) myricetin, (8) quercetin and (9) morin.

Finally, with the addition of acetonitrile or methanol at concentrations up to 20%, no further improvement in the peak resolution of these selected dyes was observed. One negative effect of adding increasing amounts of modifiers, was the suppression of the fluorescence signals of the dyes. Therefore, when using fluorescence detection, lower concentrations of modifiers (5%) are recommended. In order to resolve the aforementioned peaks, in the following section the effect of voltage was investigated.

3.4.9 The effect of voltage on separation at pH= 9.2

To examine the effect of voltage, 4 different voltages 18, 20, 22 and 25 kV were used for the separation of the mixture of 10 dyes. In these experiments either 5% acetonitrile or 5% methanol were added to the 20 mM borate buffer and 20 mM SDS at pH= 9.2. **Figure 3.32** shows the separation of the mixture of dyes using different voltages without modifier.

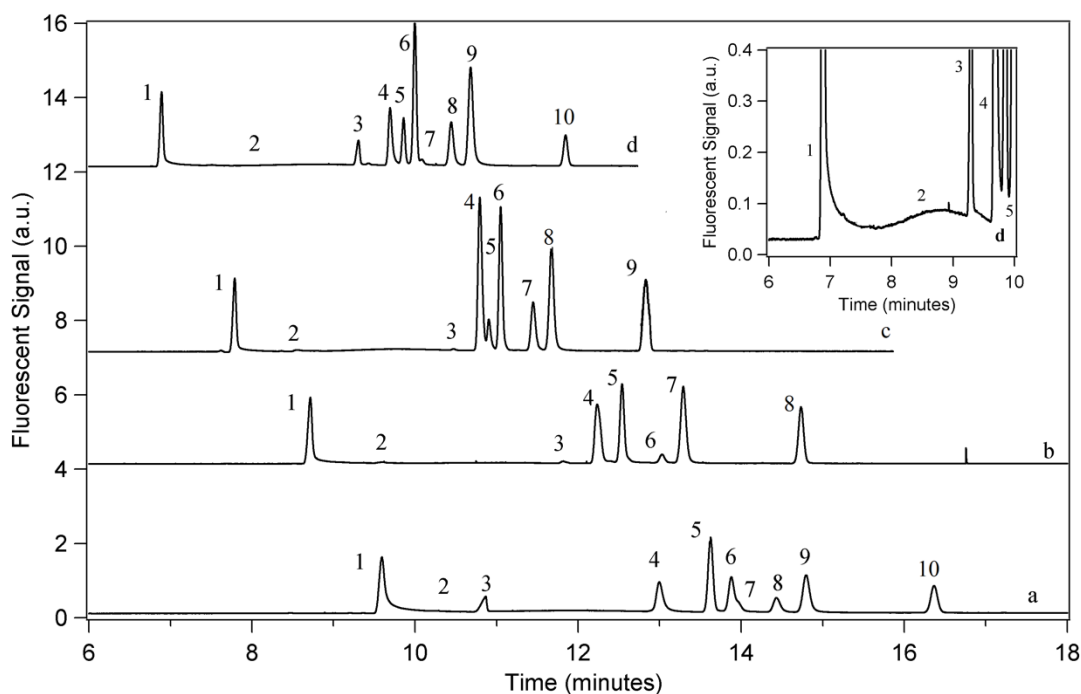


Figure 3.32 Separation of selected anthraquinones and flavonoid dyes ($10 \mu\text{g mL}^{-1}$ alizarin, $3 \mu\text{g mL}^{-1}$ purpurin, $1 \mu\text{g mL}^{-1}$ emodin, $0.5 \mu\text{g mL}^{-1}$ kaempferol, $1 \mu\text{g mL}^{-1}$ apigenin, $2 \mu\text{g mL}^{-1}$ luteolin, $10 \mu\text{g mL}^{-1}$ carmine, $2 \mu\text{g mL}^{-1}$ myricetin, $1 \mu\text{g mL}^{-1}$ quercetin, $0.5 \mu\text{g mL}^{-1}$ morin) in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, at: (a) 18 kV, (b) 20 kV, (c) 22 kV and (d) 25 kV. The assigned peaks for (a): (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin, (6) luteolin, (7) carmine, (8) myricetin, (9) quercetin and (10) morin. Run (b): (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin/ luteolin/ carmine, (6) myricetin, (7) quercetin and (8) morin. Run (c): (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) luteolin, (6) apigenin/ carmine, (7) myricetin, (8) quercetin and (9) morin. Run (d): (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) luteolin, (6) apigenin, (7) carmine, (8) myricetin, (9) quercetin and (10) morin. Reproduced with permission from Ref [49]. – Copyright: MDPI AG

Peak 5 in **Figure 3.32-b** was assigned as the co-elution of luteolin and apigenin. The comparison between the runs showed that in the absence of modifier, by increasing the voltage, the peak resolution of luteolin (5) and apigenin (4) was improved (run c and d), but the baseline separation of carmine was not achieved. The results of the effect of different voltages in the presence of 5% acetonitrile are presented in **Figure 3.33**. It was concluded that using the highest voltage (25 kV, Figure 3.33-d) in the presence of 5% acetonitrile was the most useful voltage for resolving luteolin (5) and apigenin (6).

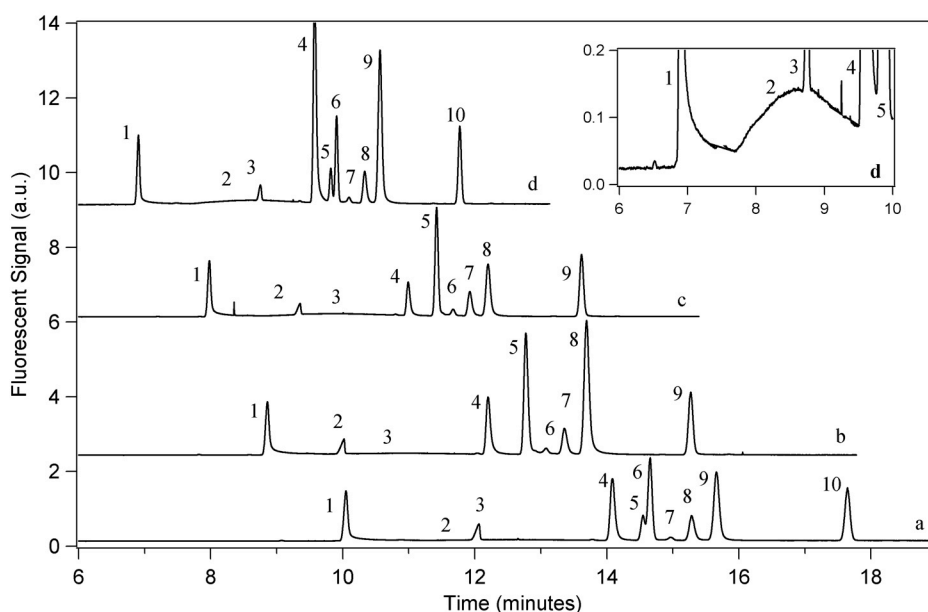


Figure 3.33 Separation of selected anthraquinones and flavonoid dyes (the same concentrations used in Figure 3.32), in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, using 5% (v/v) of acetonitrile, at: **(a)** 18 kV, **(b)** 20 kV **(c)** 22 kV and **(d)** 25 kV The assigned peaks for **(a)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) luteolin, (6) apigenin, (7) carmine, (8) myricetin, (9) quercetin and (10) morin. Runs **(b, c)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) luteolin/apigenin, (6) carmine, (7) myricetin, (8) quercetin and (9) morin. Run **(d)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) luteolin, (6) apigenin, (7) carmine, (8) myricetin, (9) quercetin and (10) morin. Reproduced with permission from Ref [49]. – Copyright: MDPI AG.

Similar experiments were carried out using the same applied voltages in the presence of 5% methanol (**Figure 3.34**). The comparison of run ‘a’, ‘b’, and ‘c’ indicated that using different voltage in the presence of methanol, did not resolve the co-elution of carmine and luteolin, but did result in baseline separation of apigenin (5) and luteolin (6). It was observed that using 25 kV applied voltage caused the overlap of apigenin, luteolin and carmine (5).

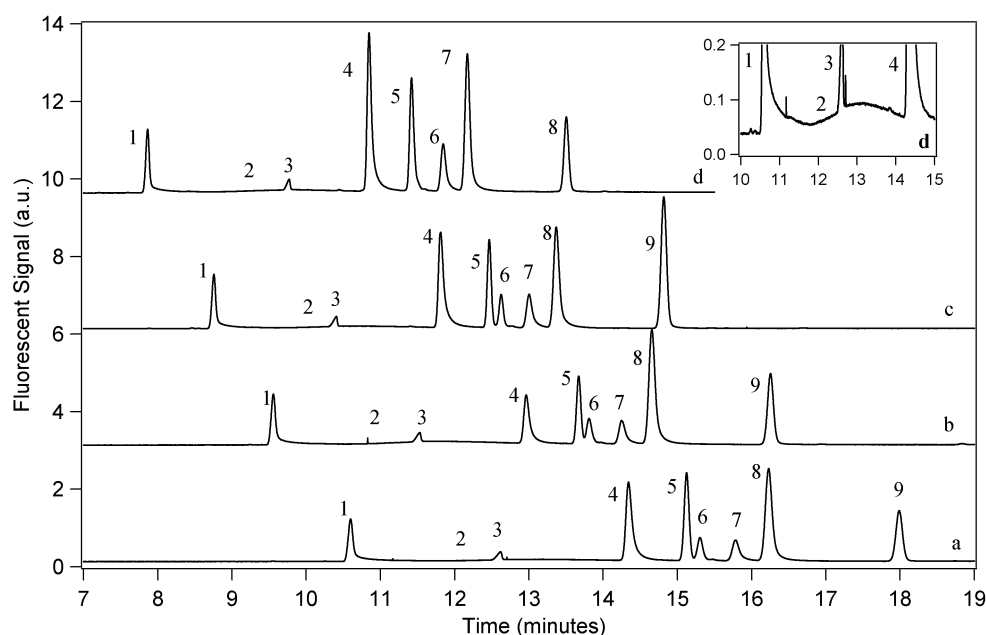


Figure 3.34 Separation of selected anthraquinones and flavonoid dyes (the same concentrations used in Figure 3.29), in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, using 5% (v/v) of methanol, at: **(a)** 18 kV, **(b)** 20 kV, **(c)** 22 kV and **(d)** 25 kV. The assigned peaks for **(a)**, **(b)** and **(c)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) apigenin, (6) luteolin/carmine, (7) myricetin, (8) quercetin and (9) morin. Run **(d)**: (1) alizarin, (2) purpurin, (3) emodin, (4) kaempferol, (5) luteolin/apigenin/ carmine, (6) myricetin, (7) quercetin and (8) morin.

3.4.10 Identification of dyes at pH= 9.2

In order to identify dyes at pH= 9.2 several experiments were performed and the results are presented in **Figure 3.35** – **Figure 3.40**. In run (a) and (b) kaempferol (1) eluted first, and

in run (c) apigenin (1) eluted before luteolin (2). Therefore, the elution order of 3 flavonoids in run (d) was defined as: kaempferol (1), apigenin (2) and luteolin (3).

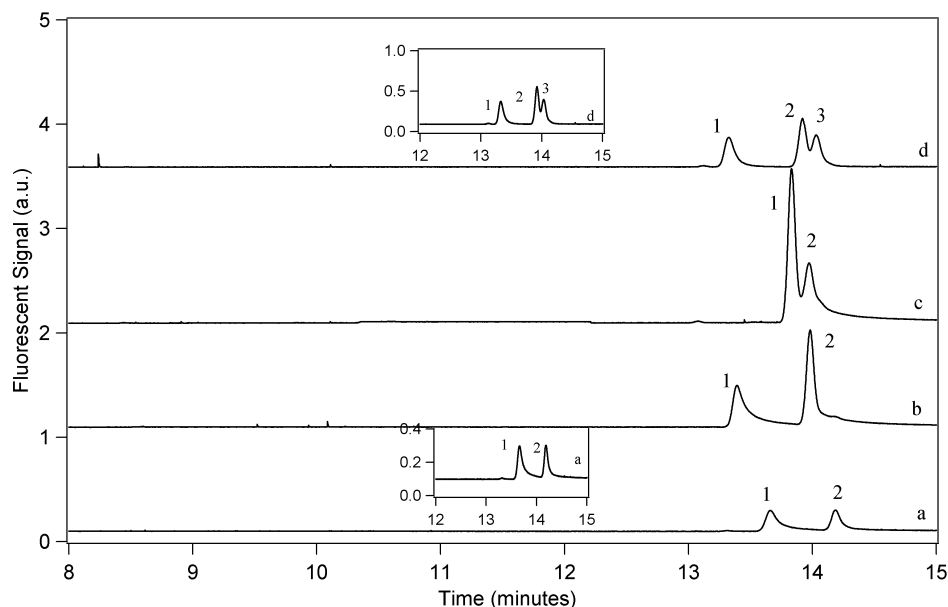


Figure 3.35 Separation of a mixture of ($1 \mu\text{g ml}^{-1}$ kaempferol, $5 \mu\text{g ml}^{-1}$ luteolin, $1 \mu\text{g ml}^{-1}$ apigenin, in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, using an applied potential of 18 kV. Peak identities: run (a) (1) kaempferol, (2) luteolin, run (b): (1) kaempferol, (2) apigenin, run (c): (1) apigenin, (2) luteolin, and run (d): (1) kaempferol, (2) apigenin (2) and (3) luteolin.

The results of studying the elution order of luteolin, apigenin, and quercetin are presented in (Figure 3. 36). It was observed that the elution order of apigenin differed, where in run (d) and (e) it eluted after or before luteolin. This study showed that in the mixture of these 3 dyes, quercetin eluted last and the elution order was defined as: luteolin/ apigenin, quercetin

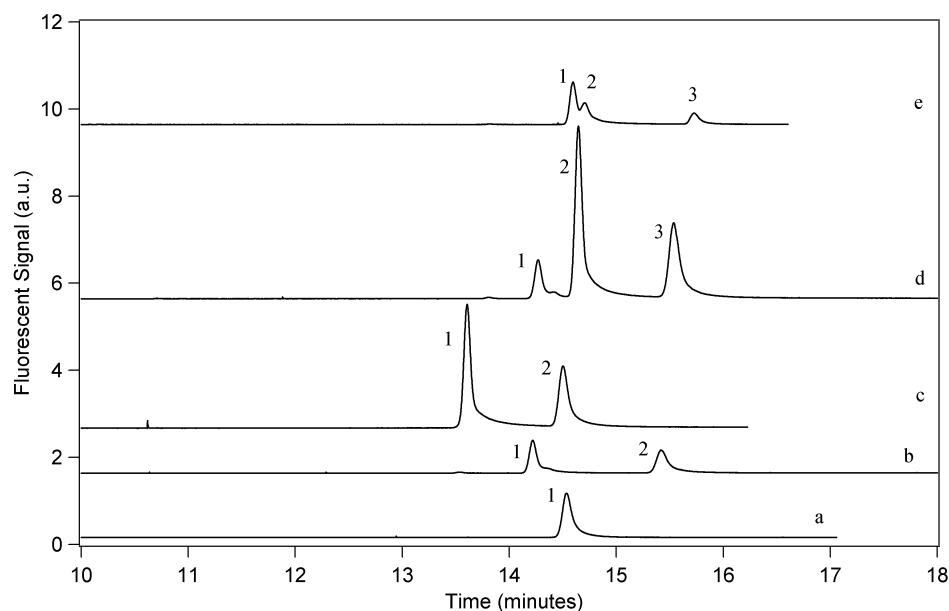


Figure 3.36 Separation of a mixture of flavonoids in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, using an applied potential of 18 kV. Peak identities: run **(a)**: (1) $2 \mu\text{g ml}^{-1}$ quercetin, run **(b)**: (1) $5 \mu\text{g ml}^{-1}$ luteolin, (2) $1 \mu\text{g ml}^{-1}$ quercetin, run **(c)**: (1) $1 \mu\text{g ml}^{-1}$ apigenin, (2) $1 \mu\text{g ml}^{-1}$ quercetin. Run **(d)**: (1) $5 \mu\text{g ml}^{-1}$ luteolin, (2) $5 \mu\text{g ml}^{-1}$ apigenin, (3) $5 \mu\text{g ml}^{-1}$ quercetin, run **(e)**: (1) $1 \mu\text{g ml}^{-1}$ apigenin, (2) $1 \mu\text{g ml}^{-1}$ luteolin (3) $1 \mu\text{g ml}^{-1}$ quercetin

Next, the elution order of emodin, kaempferol and myricetin was explored (**Figure 3.37**). The comparison of run (a) and (b) shows that kaempferol (1) eluted prior to myricetin (2), and when emodin was added it eluted earlier than kaempferol. The order of elution was defined as: emodin (1), kaempferol (2), myricetin (3).

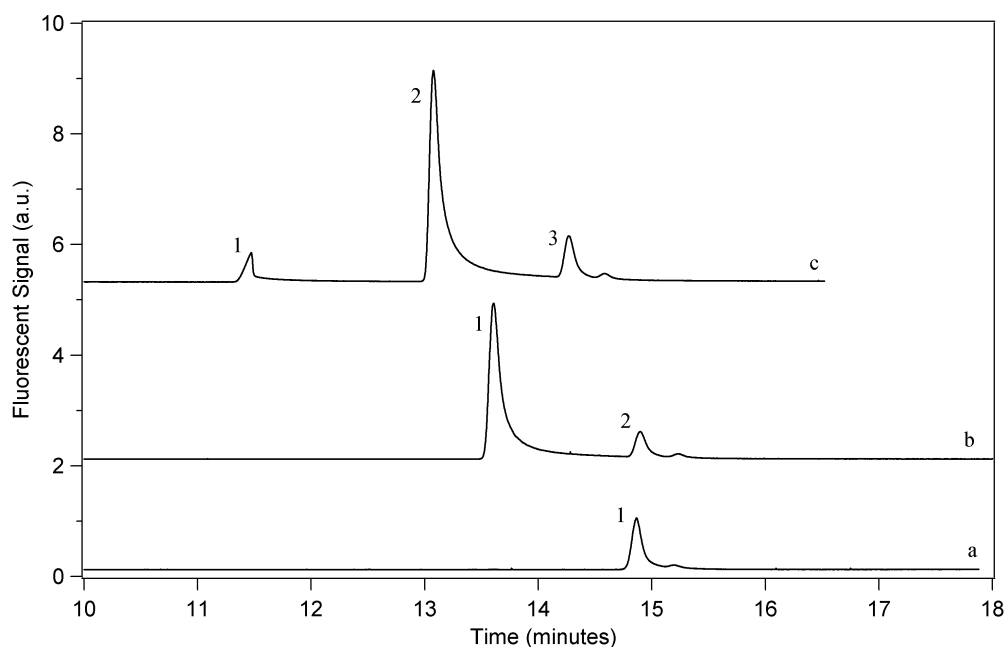


Figure 3.37 Separation of a mixture of $3 \mu\text{g mL}^{-1}$ myricetin, kaempferol, emodin in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, using an applied potential of 18 kV. Peak identities: run (a): (1) myricetin, run (b): (1) kaempferol, (2) myricetin, and run (c): (1) emodin, (2) kaempferol, and (3) myricetin.

It is worth noting that at pH= 9.2 emodin (1) had a distorted peak which was possibly due to differences in the conductivity of the analyte ($3 \mu\text{g mL}^{-1}$) and the carrier electrolyte [55]. By decreasing the pH to 8.0 the emodin peak was more Gaussian in shape indicating that conductivity in the analyte band was no longer higher than the running buffer under these conditions (Figure 3.17-f peak 3).

Figure 3.38 shows the separation of the same mixture of dyes including apigenin and quercetin. The findings from **Figure 3.32** and **Figure 3.33** established the elution of apigenin (3) after kaempferol (2) and quercetin (5) later. In order to assign the peaks, the concentration of kaempferol (2) and myricetin (4) were increased and resulted in greater signals in run (b). These findings helped to define the elution order of dyes as: emodin (1), kaempferol (2), apigenin (3), myricetin (4) and quercetin (5).

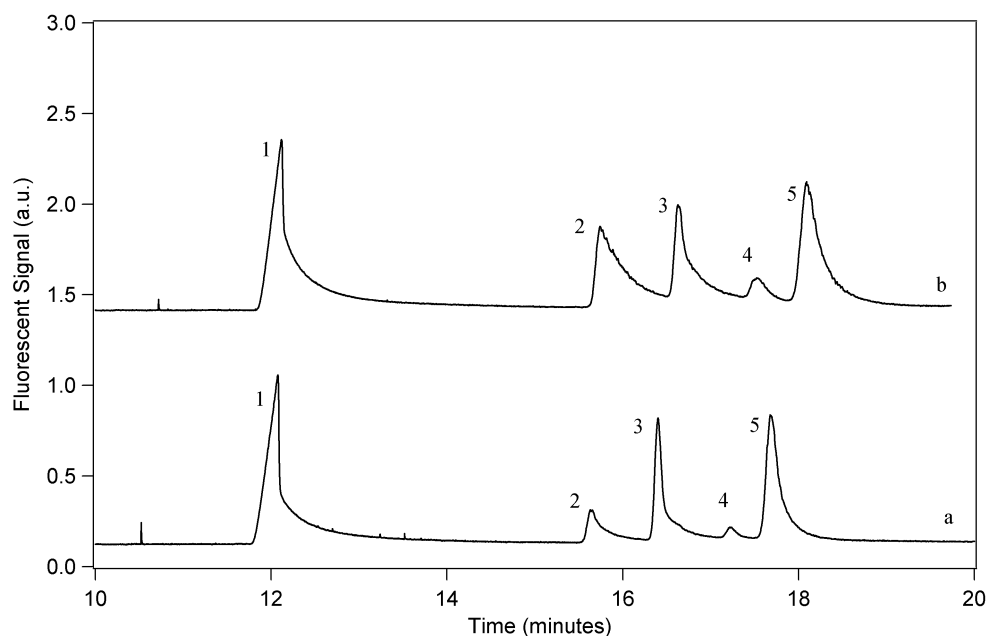


Figure 3.38 Separation of a mixture of dyes in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, using an applied potential of 18 kV. Peak identities: **run (a):** (1) $10 \mu\text{g ml}^{-1}$ emodin, (2) kaempferol $1 \mu\text{g ml}^{-1}$, (3) $1 \mu\text{g ml}^{-1}$ apigenin, (4) $5 \mu\text{g ml}^{-1}$ myricetin, (5) $5 \mu\text{g ml}^{-1}$ quercetin. **Run (b):** (1) $10 \mu\text{g ml}^{-1}$ emodin, (2) $3 \mu\text{g ml}^{-1}$ kaempferol, (3) $1 \mu\text{g ml}^{-1}$ apigenin, (4) $10 \mu\text{g ml}^{-1}$ myricetin, (5) $5 \mu\text{g ml}^{-1}$ quercetin.

Figure 3.39 presents the identification of the mixture of dyes where in each run one dye was added to the mixture. Run (a) shows the baseline separation of alizarin (1), emodin (2) and morin (3). Luteolin (3) was added to the mixture in run (b) and it eluted after emodin (2). Run (c) shows that apigenin (4) was added to the mixture used in run (b) and it eluted after luteolin (3). The same mixture of dyes including kaempferol (3) was injected in run (d) and by decreasing the amount of kaempferol in run (e) its peaks decreased. Therefore it was assigned between emodin (2) and luteolin (4). Quercetin (6) was added to the same mixture of dyes used in run (d). The results from run (e) show that quercetin eluted before morin (7). The concentration of emodin, kaempferol and luteolin was increased in run (f) and peak (2) was an overlap of these 3 dyes. Peak 3, 4 and 5 were assigned as: apigenin,

quercetin and morin. From this study the elution order of dyes was defined as: alizarin, emodin, kaempferol, luteolin, apigenin, quercetin, morin.

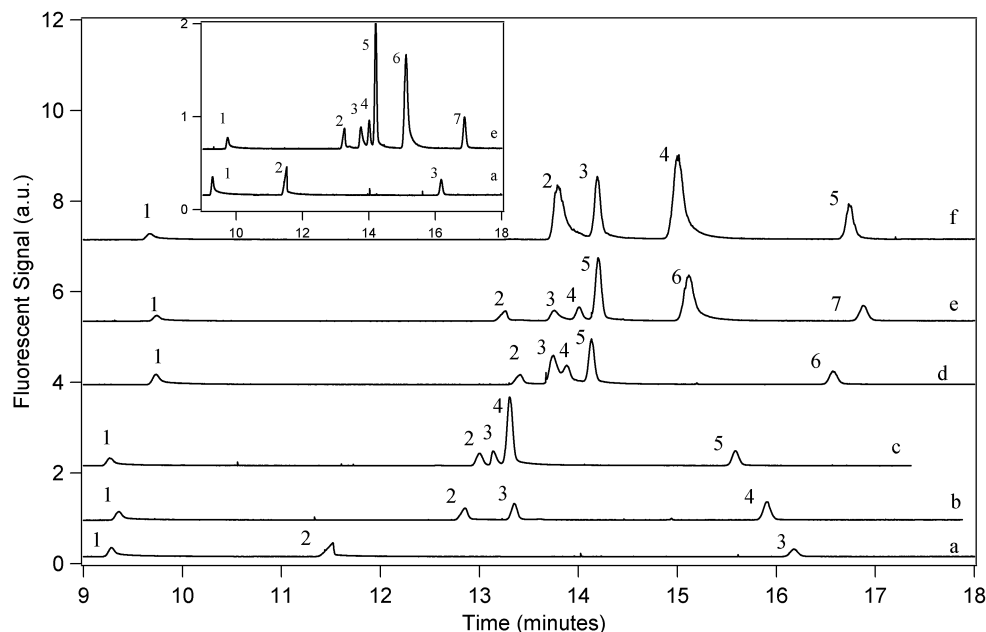


Figure 3.39 Separation of a mixture of dyes in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, using an applied potential of 18 kV. Peak identities: run **(a)**: (1) 5 $\mu\text{g ml}^{-1}$ alizarin, (2) 5 $\mu\text{g ml}^{-1}$ emodin, (3) 0.5 $\mu\text{g ml}^{-1}$ morin. Run **(b)**: (1) 5 $\mu\text{g ml}^{-1}$ alizarin, (2) 5 $\mu\text{g ml}^{-1}$ emodin, (3) 5 $\mu\text{g ml}^{-1}$ luteolin, (4) 0.5 $\mu\text{g ml}^{-1}$ morin. Run **(c)**: (1) 5 $\mu\text{g ml}^{-1}$ alizarin, (2) 5 $\mu\text{g ml}^{-1}$ emodin, (3) 5 $\mu\text{g ml}^{-1}$ luteolin, (4) 5 $\mu\text{g ml}^{-1}$ apigenin, (5) 0.5 $\mu\text{g ml}^{-1}$ morin. Run **(d)**: (1) 5 $\mu\text{g ml}^{-1}$ alizarin, (2) 5 $\mu\text{g ml}^{-1}$ emodin, (3) 6 $\mu\text{g ml}^{-1}$ kaempferol, (4) 5 $\mu\text{g ml}^{-1}$ luteolin, (6) 3 $\mu\text{g ml}^{-1}$ apigenin and (7) 0.5 $\mu\text{g ml}^{-1}$ morin. Run **(e)**: (1) 5 $\mu\text{g ml}^{-1}$ alizarin, (2) 5 $\mu\text{g ml}^{-1}$ emodin, (3) 3 $\mu\text{g ml}^{-1}$ kaempferol, (4) 5 $\mu\text{g ml}^{-1}$ luteolin, (5) 4 $\mu\text{g ml}^{-1}$ apigenin, (6) 5 $\mu\text{g ml}^{-1}$ quercetin, and (7) 0.5 $\mu\text{g ml}^{-1}$ morin. Run **(f)**: (1) 5 $\mu\text{g ml}^{-1}$ alizarin, (2) 7 $\mu\text{g ml}^{-1}$ emodin, 5 $\mu\text{g ml}^{-1}$ kaempferol, 6 $\mu\text{g ml}^{-1}$ luteolin, (3) 4 $\mu\text{g ml}^{-1}$ apigenin, (4) 10 $\mu\text{g ml}^{-1}$ quercetin, (5) 0.5 $\mu\text{g ml}^{-1}$ morin.

The effect of concentration of kaempferol and luteolin on overlapping was explored and the results are given in **Figure 3.40**. A mixture of 3 $\mu\text{g ml}^{-1}$ kaempferol (1), 3 $\mu\text{g ml}^{-1}$ luteolin (2) and 1 $\mu\text{g ml}^{-1}$ apigenin (3) was injected (run a) and it was noticed luteolin was eluted as a shoulder peak of kaempferol. The amount of kaempferol and luteolin was decreased by one third in run (b) and the peak shape of luteolin was improved but the baseline

separation was not achieved. The amount of kaempferol was decreased by half in run (c) and it resulted in 2 separate peaks of kaempferol (1) and luteolin (2).

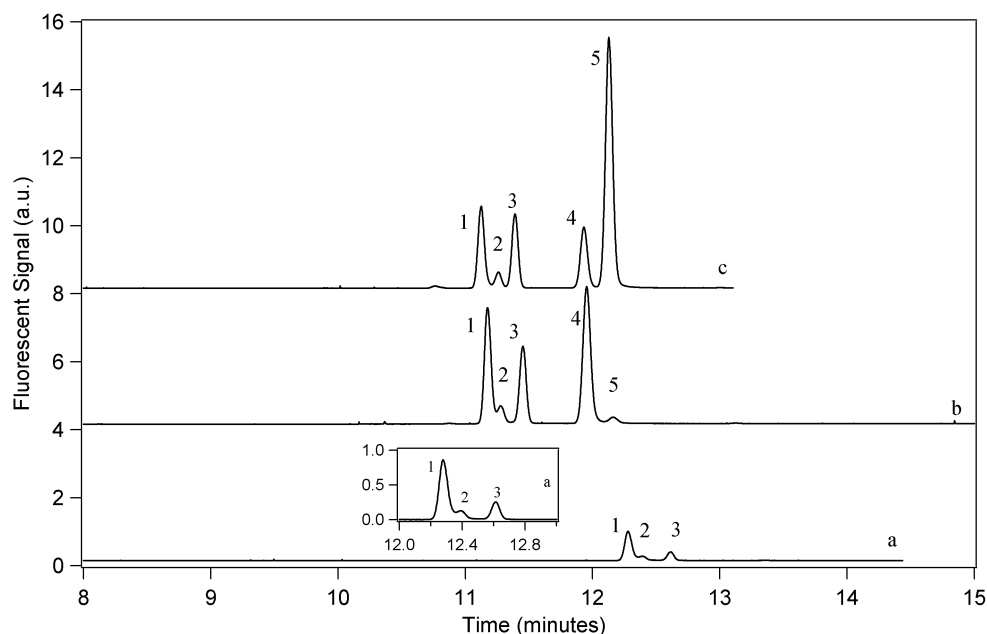


Figure 3.40 Separation of a mixture of dyes in a 20 mM borate buffer and 20 mM SDS at pH= 9.2, using an applied potential of 18 kV. Peak identities: run **(a)**: (1) $1 \mu\text{g ml}^{-1}$ kaempferol, (2) $3 \mu\text{g ml}^{-1}$ luteolin and (3) $1 \mu\text{g ml}^{-1}$ apigenin; run **(b)**: (1) $1 \mu\text{g ml}^{-1}$ kaempferol, (2) $1 \mu\text{g ml}^{-1}$ luteolin, (3) $1 \mu\text{g ml}^{-1}$ apigenin, (4) $5 \mu\text{g ml}^{-1}$ myricetin, (5) $1 \mu\text{g ml}^{-1}$ quercetin; run **(c)**: (1) $0.5 \mu\text{g ml}^{-1}$ kaempferol, (2) $1 \mu\text{g ml}^{-1}$ luteolin, (3) $1 \mu\text{g ml}^{-1}$ apigenin, (4) $2 \mu\text{g ml}^{-1}$ myricetin, (5) $5 \mu\text{g ml}^{-1}$ quercetin.

In this study the elution order of dyes was investigated. $5 \mu\text{g ml}^{-1}$ myricetin and $1 \mu\text{g ml}^{-1}$ quercetin were added to the mixture of dyes (run a). The amount of quercetin and myricetin were changed to $5 \mu\text{g ml}^{-1}$ and $2 \mu\text{g ml}^{-1}$ respectively in run (c). The comparison of the peak sizes in these 2 runs showed that peak (4) and (5) belonged to myricetin and quercetin respectively.

The elution order for these dyes was defined as: kaempferol, luteolin, apigenin, myricetin, and quercetin. To this point, it can be concluded that the base line separation of most dyes was achieved at pH= 8.5. From **Figures 3.2, 3.4 and 3.7** it can also be concluded that the

fluorescence intensity of dyes was affected by pH. Therefore, in next section the detection limits of all dyes at pH values used in this study: 8.0, 8.5 and 9.2 were calculated.

3.5 The detection limits for the dyes

Apart from separation properties of the CE-LIF instrument, detection limits were also determined for these dyes. Achieving low detection limits was important for this work prior to using this approach for identifying dyes in field samples. Furthermore, because of the small sample size requirement for CE separations, a low detection limit is also desirable for minimizing damage to potentially valuable samples such as museum objects. Detection limits for the dyes used in this study are shown in **Table 3.1**. For these separations, a 18 kV and 20 mM borate buffer at pH = 8.0, 8.5 and 9.2. Detection limits were calculated by determining 3s of the background signal ($n > 500$) and by comparing the magnitude of the background signal to the signal of a standard of known concentration ($5 \mu\text{g ml}^{-1}$ except for apigenin $0.5 \mu\text{g ml}^{-1}$). Absolute detection limits (pg) were calculated by multiplying the volume injected by the concentration of the dye. The volume of each dye injected can be determined using the equation *Eq 3.1* [56]:

$$V_{inj} = V_{cap} \times (t_{inj} \div t_m) \times (E_{inj} \div E_m) \quad \text{Eq 3.1}$$

Where:

- V_{inj} = volume of sample injected (L)
- V_{cap} = volume of capillary (L)
- t_{inj} = time that sample introduced in to capillary (s)
- t_m = migration time of the dye (s)
- E_{inj} = voltage applied for introduction of the sample into the capillary (V)
- E_m = the voltage applied for migration of the dye in the capillary (V)

For some dyes such as alizarin, changing the pH had little or no benefit, whereas for purpurin, the lower pH improved detection limits significantly which could be due to improvements in peak shape and an increase in peak height, without a significant increase in background noise.

Table 3.1 Detection limits of selected anthraquinone and flavonoid dyes

Dyes	pH = 8.0		pH = 8.5		pH = 9.2	
	ng ml ⁻¹	pg	ng ml ⁻¹	pg	ng ml ⁻¹	pg
Alizarin	0.066	0.017	0.019	0.004	0.061	0.011
Purpurin	0.222	0.055	0.195	0.040	N/A	N/A
Emodin	0.044	0.010	0.054	0.011	0.057	0.009
Carmine	0.170	0.036	0.204	0.033	0.636	0.088
Apigenin	0.005	0.001	0.030	0.005	0.006	0.001
Ap-7- <i>O</i> -glu	0.064	0.014	0.149	0.033	0.680	0.128
Luteolin	0.028	0.006	0.018	0.003	0.049	0.005
Lu7- <i>O</i> -glu	0.011	0.003	0.012	0.003	0.090	0.017
Kaempferol	0.008	0.002	0.049	0.008	0.014	0.001
Quercetin	0.003	0.001	0.129	0.019	0.173	0.017
Morin	0.002	0.001	0.075	0.010	0.010	0.001
Myricetin	0.011	0.002	0.108	0.016	0.091	0.009

Absolute limits of detection for most of these dyes was less than 0.1 pg except for ap-7-*O*-glu at pH= 9.2 which was 0.128 pg. The comparison of changes in anthraquinones limit of detections (LOD) it can be concluded that except for carmine that showed the lowest LOD at pH=8.0, for anthraquinones improved limit of detections were achieved at pH = 8.5.

Regarding flavones, the LOD of glucoside forms of apigenin and luteolin slightly improved by decreasing the pH. From **Table 3.1** it can be concluded that pH=8.5 is the optimum pH to have the lowest LOD for flavonoids. In case of flavonols, LOD was improved by lowering the pH except for kaempferol which showed the highest detection limit at pH=8.5. However, the best LODs for flavonols was pH=8.0, but comparison the baseline separation of these dyes (**Figure 3.7**), it can be concluded that pH=8.5 would be the best pH for CE separation.

3.6 Conclusion

It was observed that separation and identification of a limited number of dyes from the same class was not complicated. However, it was challenging when the separation and identification of a group of dyes from different classes were studied. Generally speaking, an increase in the number of dyes, increases the chance of having compounds with the same or close mobility lead to co-elution. Therefore, the key parameters in separation: pH, voltage and modifier should be manipulated to the extent that the baseline separation of all dyes is achieved in the shortest time.

The first approach was the study of baseline separation and elution order of the dyes belonged to the same class at different pH values = 8.0, 8.5 and 9.2. Considering the peak shapes of anthraquinones: alizarin, purpurin, emodin and carmine the best baseline separation was achieved at pH= 8.5. It was also observed that at pH= 9.2, there was no peak for purpurin due to its instability and possible photo-decomposition at alkaline pH. The elution order of anthraquinones did not change at different pH values. Regarding flavones: apigenin, ap-7-*O*-glu, luteolin, and lu-7-*O*-glu, the results proved that pH= 8.5 was the best pH

to achieve the baseline separation. Concerning the elution order of these dyes it was noticed that at pH= 8.0 and 8.5 lu-7-*O*-glu eluted prior to ap-7-*O*-glu with about 2 and 1 minute difference, respectively. At pH= 9.2 ap-7-*O*-glu eluted prior to lu-7-*O*-glu. The elution order of apigenin and luteolin was also shifting with changing pH as: luteolin eluted prior to apigenin at pH = 8.5 and 9.2 and after at pH= 8.0. It was observed that apigenin and luteolin showed similar mobility at pH= 9.2 and they easily displaced in different runs. This caused difficulty in assigning these peaks. Regarding flavonols: kaempferol, myricetin, quercetin and morin by compromising the run time and the best baseline separation, it can be concluded that the best pH for separation of flavonols was pH= 8.5. The results from this study proved that the elution order of flavonols did not change significantly by changing the pH values.

Another objective of this study was employing CE-LIF for separation and identification of the mixture of dyes belonged to different classes. In textiles the desired hue is often gained by mixing different colourants, e.g. the green colour was the result of mixing indigo and weld. Therefore, separation of the mixture of anthraquinones and flavonoids was studied. The separation of fresh solution of the mixture of dyes was studied at different pH values: 8.0, 8.5 and 9.2. It was noticed at pH= 8.0, 2 peaks were missed and it was due to the co-elution of myricetin with carmine and quercetin with morin. To resolve these peaks, the effect of acetonitrile and methanol at 5% and 10% (v/v) was investigated. It was concluded that at pH= 8.0 different concentrations of acetonitrile and methanol did not improve separation of these peaks. The effect of applied voltage (18-25 kV) on the separation with and without modifiers was studied. The results proved that different voltages in the presence of modifiers at pH= 8.0 could not improve the baseline separation of myricetin

and carmine as well as quercetin and morin. Using 5% methanol at 20 kV showed a minor impact where the peak belonging to quercetin eluted as a shoulder between myricetin and morin.

The same systematic study was applied at pH=8.5 and 9.2. Separation at pH=8.5 resulted in co-elution of kaempferol with emodin, apigenin with myricetin and carmine, and luteolin with quercetin. Using 5% (v/v) acetonitrile could resolve the peaks. While, using 5% methanol did not allow the resolution of the peaks, but using 10% methanol the baseline separation of most dyes was achieved except for purpurin, kaempferol, and emodin. The effect of applied voltage on the separation of these dyes with and without modifier was explored. The best baseline separation for most dyes especially carmine was achieved at 20 kV, and emodin eluted as a shoulder peak of kaempferol. The results of separation of dyes at different voltages using 5% (v/v) acetonitrile proved that the best baseline separation considering peak shapes and elution time was achieved at 22 kV. The effect of 5% (v/v) methanol at different voltages showed that at 18 kV emodin eluted as a shoulder peak of apigenin and it superimposed carmine. The best baseline separation of dyes was achieved using 20 kV and 22 kV, except for emodin, which eluted at the same time with purpurin. Separation at pH= 9.2 resulted in co-elution of apigenin with luteolin, and the peak belonged to purpurin could not be detected due to its instability at alkaline. Using 5% (v/v) acetonitrile could resolve apigenin and luteolin. While increasing acetonitrile did not help it caused that the peak of luteolin eluted as a shoulder with apigenin peak. The effect of 5% and 10% (v/v) methanol was studied on the separation of the mixture of dyes. It was noticed that in the presence of methanol the peak belonged to carmine shifted toward lute-

olin/apigenin. Using 5% (v/v) methanol could resolve luteolin from apigenin but By increasing methanol 10% (v/v) the co-elution of luteolin and apigenin happened and did not help for baseline separation of carmine. The effect of different voltages (18-25 kV) without and with 5% acetonitrile and 5% methanol was studied. In the absence of modifier, by increasing the voltage, the peak resolution of luteolin and apigenin was improved but the baseline separation of carmine was not achieved. The results of the effect of different voltages in the presence of 5% acetonitrile showed that using the highest voltage 25 kV was the most useful voltage for resolving luteolin and apigenin. Using different voltages in the presence of 5% (v/v) methanol, did not resolve the co-elution of carmine and luteolin, but did result in baseline separation of apigenin (5) and luteolin (6). It was observed that using 25 kV applied voltage caused the overlap of apigenin, luteolin and carmine (5). The elution order of dyes from the same class in the mixture of dyes showed the same order observed for each of them except for kaempferol at pH= 8.0. However, the elution order of a group of 4 flavonols, at pH= 8.0, 8.5 and 9.2 were the same: kaempferol, myricetin, quercetin and morin. These dyes showed the same elution order in the mixture of dyes at pH= 9.2 and 8.5 except kaempferol eluted last before apigenin. The comparison of the dyes eluting close to kaempferol at all pH conditions indicated that kaempferol eluted close to apigenin. Therefore, the change of the elution order of kaempferol at pH= 8.0 may be due to the interaction between kaempferol and apigenin.

Defining the best separation condition is a challenging task since by manipulating the separation factors one issue was solved while another one was raised. For instance, at pH= 8.5 the co-elution of luteolin and quercetin as well as carmine and myricetin were observed. However, by decreasing the pH to 8.0 luteolin and quercetin were separated but the co-

elution of quercetin and morin as well as carmine and myricetin were observed. Using different voltages and modifiers did not resolve peaks at pH= 8.0. It is also worth noting that the fluorescence properties of dyes are affected by pH. Also, the separation of purpurin using CE-LIF under alkaline condition can be complicated by reproducibility in peak height and peak shape. Therefore, selecting a pH is a compromise between detectable fluorescence signals and the baseline separation of dyes. It was concluded that the base line separation of all dyes was at pH= 8.5 using 5% acetonitrile and applied voltage of 22 kV, where also resulted in a well-shaped peak for purpurin.

The interest in using MEKC with LIF for detection is driven by the need to develop minimally destructive methods for identifying dyes in historical objects such as textiles. The most important advantage of using LIF is that the low detection limits and high sensitivity allow for the separation of very small quantities of sample, which makes this approach suitable for working with valuable historical objects and the results from the detection limits of dyes proved this hypothesis.

3.7 References

1. Cardon, D., *Natural dyes: sources, tradition, technology and science*. 2007: Archetype.
2. Bechtold, T. and R. Mussak, *Handbook of natural colorants*. 2009: John Wiley & Sons.
3. Peggie, D.A., *The development and application of analytical methods for the identification of dyes on historical textiles*. 2006, University of Edinburgh.
4. Degano, I., et al., *Analytical Methods for the Characterization of Organic Dyes in Artworks and in Historical Textiles*. *Applied Spectroscopy Reviews*, 2009. **44**(5): p. 363-410.
5. De la Rie, E.R., *Fluorescence of paint and varnish layers (Part II)*. *Studies in Conservation*, 1982. **27**(2): p. 65-69.
6. Wallert, A., *Unusual pigments on a Greek marble basin*. *Studies in Conservation*, 1995. **40**(3): p. 177-188.
7. Miliani, C., A. Romani, and G. Favaro, *A spectrophotometric and fluorimetric study of some anthraquinoid and indigoid colorants used in artistic paintings*. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 1998. **54**(4): p. 581-588.
8. Clementi, C., et al., *In-situ fluorimetry: A powerful non-invasive diagnostic technique for natural dyes used in artefacts: Part II. Identification of orcein and indigo in Renaissance tapestries*. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 2009. **71**(5): p. 2057-2062.
9. Low, M.J. and N.S. Baer, *Application of Infrared Fourier Transform Spectroscopy to Problems in Conservation. I. General Principles*. *Studies in conservation*, 1977. **22**(3): p. 116-128.
10. Shearer, J.C., et al., *FTIR in the service of art conservation*. *Analytical Chemistry*, 1983. **55**(8): p. 874A-880A.
11. Gillard, R., et al., *The detection of dyes by FTIR microscopy*. *Studies in Conservation*, 1994. **39**(3): p. 187-192.
12. Edwards, H., *Illumination of a mediaeval mystery: the FT-Raman spectroscopic analysis of red pigment from a mediaeval corbel in the church St Clement of Rome, Fiskerton*. *Journal of molecular structure*, 2003. **661**: p. 271-277.
13. Edwards, H.G., S.E. Jorge Villar, and K.A. Eremin, *Raman spectroscopic analysis of pigments from dynastic Egyptian funerary artefacts*. *Journal of Raman spectroscopy*, 2004. **35**(8-9): p. 786-795.
14. Kharbade, B. and O. Agrawal, *Analysis of natural dyes in Indian historic textiles*. *Studies in conservation*, 1988: p. 1-8.
15. Scott, D.A., et al., *Technical examination of a fifteenth-century German illuminated manuscript on paper: a case study in the identification of materials*. *Studies in Conservation*, 2001: p. 93-108.
16. Wouters, J. and A. Verhecken, *The coccid insect dyes: HPLC and computerized diode-array analysis of dyed yarns*. *Studies in Conservation*, 1989. **34**(4): p. 189-200.

17. Colombini, M.P., et al., *Integrated analytical techniques for the study of ancient Greek polychromy*. Talanta, 2004. **63**(4): p. 839-48.
18. Doménech-Carbó, M., et al., *Analytical study of canvas painting collection from the Basilica de la Virgen de los Desamparados using SEM/EDX, FT-IR, GC and electrochemical techniques*. Fresenius' journal of analytical chemistry, 2001. **369**(7-8): p. 571-575.
19. Puchalska, M., et al., *Identification of anthraquinone coloring matters in natural red dyes by electrospray mass spectrometry coupled to capillary electrophoresis*. Journal of mass spectrometry, 2003. **38**(12): p. 1252-1258.
20. Surowiec, I., et al., *Capillary electrophoretic determination of main components of natural dyes with MS detection*. J Sep Sci, 2008. **31**(13): p. 2457-62.
21. Trojanowicz, M., L. Wójcik, and K. Urbaniak-Walczak, *Identification of natural dyes in historical Coptic textiles by capillary electrophoresis with diode array detection*. Chemia analityczna, 2003. **48**(3): p. 607-620.
22. Maguregui, M.I., et al., *Micellar electrokinetic chromatography method for the determination of several natural red dyestuff and lake pigments used in art work*. J Chromatogr A, 2007. **1154**(1-2): p. 429-36.
23. López-Montes, A., et al., *Simultaneous identification of natural dyes in the collection of drawings and maps from The Royal Chancellery Archives in Granada (Spain) by CE*. Electrophoresis, 2007. **28**(8): p. 1243-1251.
24. Goltz, D.M., et al., *Separation of historical dyes using capillary electrophoresis with laser-induced fluorescence detection*. Journal of Liquid Chromatography & Related Technologies, 2012. **35**(14): p. 2054-2065.
25. Ahmadi, S., D.B. Craig, and D.M. Goltz, *Micellar electrokinetic chromatography with laser-induced fluorescence detection for separation of red and yellow historical dyes*. Chromatography, 2013. **1**(1): p. 9-23.
26. Ahmadi, S., et al., *Photochemical properties of purpurin and its implications for capillary electrophoresis with laser induced fluorescence detection*. Dyes and Pigments, 2014. **105**: p. 57-62.
27. Colombini, M.P., et al., *Colour fading in textiles: A model study on the decomposition of natural dyes*. Microchemical Journal, 2007. **85**(1): p. 174-182.
28. López-Montes, A., et al., *Characterization of sepia ink in ancient graphic documents by capillary electrophoresis*. Microchemical Journal, 2009. **93**(2): p. 121-126.
29. Zlotnick, J. and F. Smith, *Separation of some black rollerball pen inks by capillary electrophoresis: preliminary data*. Forensic Science International, 1998. **92**(2): p. 269-280.
30. Svobodová, E., et al., *Use of Capillary Zone Electrophoresis and Micellar Electrokinetic Chromatography for Separations of Anthraquinone Derivatives*. Analytical Letters, 2011. **44**(10): p. 1783-1795.
31. Maguregui, M., et al., *Micellar electrokinetic chromatography method for the determination of several natural red dyestuff and lake pigments used in art work*. Journal of Chromatography A, 2007. **1154**(1): p. 429-436.
32. Lopez-Montes, A., et al., *Simultaneous identification of natural dyes in the collection of drawings and maps from The Royal Chancellery Archives in Granada (Spain) by CE*. Electrophoresis, 2007. **28**(8): p. 1243-51.

33. Craig, D., et al., *Fluorescence-based enzymatic assay by capillary electrophoresis laser-induced fluorescence detection for the determination of a few β -galactosidase molecules*. Analytical biochemistry, 1995. **226**(1): p. 147-153.
34. Wang, S.F., et al., *Study of the Electrophoretic Behaviour of Flavonoids*. Chromatographia, 2004. **59**(7-8).
35. Wang, S., et al., *Study of the electrophoretic behaviour of flavonoids*. Chromatographia, 2004. **59**(7-8): p. 507-511.
36. Khaledi, M.G., S.C. Smith, and J.K. Strasters, *Micellar electrokinetic capillary chromatography of acidic solutes: migration behavior and optimization strategies*. Analytical chemistry, 1991. **63**(17): p. 1820-1830.
37. Koyama, J., I. Toyokuni, and K. Tagahara, *Analysis of Anthraquinones by Micellar Electrokinetic Capillary Chromatography*. Phytochemical Analysis, 1997. **8**(3): p. 135-138.
38. Weng, W.C. and S.J. Sheu, *Separation of Anthraquinones by Capillary Electrophoresis and High-Performance Liquid Chromatography*. Journal of High Resolution Chromatography, 2000. **23**(2): p. 143-148.
39. Puchalska, M., et al., *Identification of anthraquinone coloring matters in natural red dyes by electrospray mass spectrometry coupled to capillary electrophoresis*. J Mass Spectrom, 2003. **38**(12): p. 1252-8.
40. Biesaga, M., *Influence of extraction methods on stability of flavonoids*. Journal of Chromatography A, 2011. **1218**(18): p. 2505-2512.
41. Mouri, C., et al., *Characterization of flavonols in plants used for textile dyeing and the significance of flavonol conjugates*. Dyes and Pigments, 2014. **100**: p. 135-141.
42. Wang, S.-P. and K.-J. Huang, *Determination of flavonoids by high-performance liquid chromatography and capillary electrophoresis*. Journal of Chromatography A, 2004. **1032**(1-2): p. 273-279.
43. Markham, K.R. and T.K. McGhie, *Separation of flavones by capillary electrophoresis: The influence of pKa on electrophoretic mobility*. Phytochemical Analysis, 1996. **7**(6): p. 300-304.
44. Herrero-Martinez, J.M., et al., *Determination of dissociation constants of flavonoids by capillary electrophoresis*. Electrophoresis, 2005. **26**(10): p. 1886-95.
45. Alvarez-Diduk, R., et al., *Deprotonation mechanism and acidity constants in aqueous solution of flavonols: a combined experimental and theoretical study*. The Journal of Physical Chemistry B, 2013. **117**(41): p. 12347-12359.
46. Rice, C. and R. Whitehead, *Electrokinetic flow in a narrow cylindrical capillary*. The Journal of Physical Chemistry, 1965. **69**(11): p. 4017-4024.
47. Jorgenson, J.W. and K.D. Lukacs, *Capillary zone electrophoresis*. Science, 1983. **222**(4621): p. 266-272.
48. McGhie, T.K., *Analysis of sugarcane flavonoids by capillary zone electrophoresis*. Journal of Chromatography A, 1993. **634**(1): p. 107-112.
49. Ahmadi, S., D. Craig, and D. Goltz, *Micellar Electrokinetic Chromatography with Laser-Induced Fluorescence Detection for Separation of Red and Yellow Historical Dyes*. Chromatography, 2013. **1**(1): p. 9-23.
50. Herrero-Martinez, J.M., et al., *Determination of flavonoid aglycones in several food samples by mixed micellar electrokinetic chromatography*. J Sep Sci, 2007. **30**(15): p. 2493-500.

51. Pietta, P., et al., *Influence of structure on the behavior of flavonoids in capillary electrophoresis*. Electrophoresis, 1994. **15**(1): p. 1326-1331.
52. Cao, Y., et al., *Analysis of flavonoids in Ginkgo biloba L. and its phytopharmaceuticals by capillary electrophoresis with electrochemical detection*. Analytical and bioanalytical chemistry, 2002. **374**(2): p. 294-299.
53. Delgado, C., et al., *Capillary electrophoresis as an alternative to HPLC for determination of honey flavonoids*. Chromatographia, 1994. **38**(1-2): p. 71-78.
54. Ng, C., et al., *Systematic optimization of micellar electrokinetic chromatographic separation of flavonoids*. Chromatographia, 1992. **34**(3-4): p. 166-172.
55. Harris, D.C., *Quantitative Chemical Analysis*. 2003: Macmillan.
56. Khaled, M.Y., M.R. Anderson, and H.M. McNair, *Micellar electrokinetic capillary chromatography of pungent compounds using simultaneous on-line ultraviolet and electrochemical detection*. Journal of chromatographic science, 1993. **31**(7): p. 259-264.

Chapter 4

Photochemical properties of purpurin and selected flavonols, effects on their separation using CE-LIF

Photochemical properties of purpurin and its implications for capillary electrophoresis with laser induced fluorescence detection

Shokoufeh Ahmadi^{1,3}, Ghodratollah Absalan², Douglas Craig³, Douglas Goltz¹

1. Department of Chemistry, University of Manitoba, Winnipeg, MB, R3T 2N2 CANADA
2. Department of Chemistry, Shiraz University, Shiraz, Iran
3. Department of Chemistry, Richardson College for the Environment and Science Complex, University of Winnipeg, 599 Portage Ave., Winnipeg, MB, R3B 2G3 CANADA

Individual contribution of authors:

Shokoufeh Ahmadi performed all analysis/separations, data collection, data interpretation as well as drafted the manuscript for publication. She made a contribution in writing the methodology and experimental sections.

Dr. Doug Goltz supervised the development of experiments and wrote discussion section of the manuscript and was the corresponding author.

Dr. Doug Craig made a contribution experimentally and helped edit the manuscript prior to publication.

Dr. Ghodratollah Absalan critically reviewed the manuscript and helped edit portions of the manuscript prior to publication.

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Photochemical properties of selected flavonol dyes: Effects on their separation using capillary electrophoresis.

Douglas Goltz^{1,2}, Shokoufeh Ahmadi^{1,2}, Jeremie Crawford^{1,2}, Douglas Craig¹.

1. Department of Chemistry, Richardson College for the Environment and Science Complex, University of Winnipeg, 599 Portage Ave., Winnipeg, MB, R3B 2G3 CANADA
2. Department of Chemistry, University of Manitoba, Winnipeg, MB, R3T 2N2 CANADA

Individual contribution of authors:

Dr. Doug Goltz supervised the development of experiments and wrote the manuscript and was the corresponding author.

Shokoufeh Ahmadi performed some analysis/separations, data collection, data interpretation as well as made a contribution in writing the methodology and experimental sections.

Jeremie Crawford performed some analysis/separations, data collection, data interpretation as well as drafted the discussion section.

Dr. Doug Craig made a contribution experimentally and helped edit the manuscript prior to publication.

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4.1 Introduction

Chapters 2 and 3 have focused on the use of MEKC with LIF for the separation of selected anthraquinone and flavonoid dyes under alkaline conditions. In the initial separation study of 4 dyes: alizarin, purpurin, carmine and morin, it was observed that the peak for purpurin was not reproducible after 60 minutes. **Figure 4.1** shows a typical separation of these dyes that were dissolved in a solution of 20 mM borate buffer with 20 mM SDS. In the initial separation (I), these dyes were prepared fresh and were electrokinetically injected to the capillary. Under these conditions all of the dyes were well separated, however the relatively large peak for purpurin appeared to have significant peak broadening. This peak broadening appeared with purpurin even when fresh samples were separated. After this separation, the column was conditioned with NaOH for 10 minutes and 20mM borate buffer for 10 minutes.

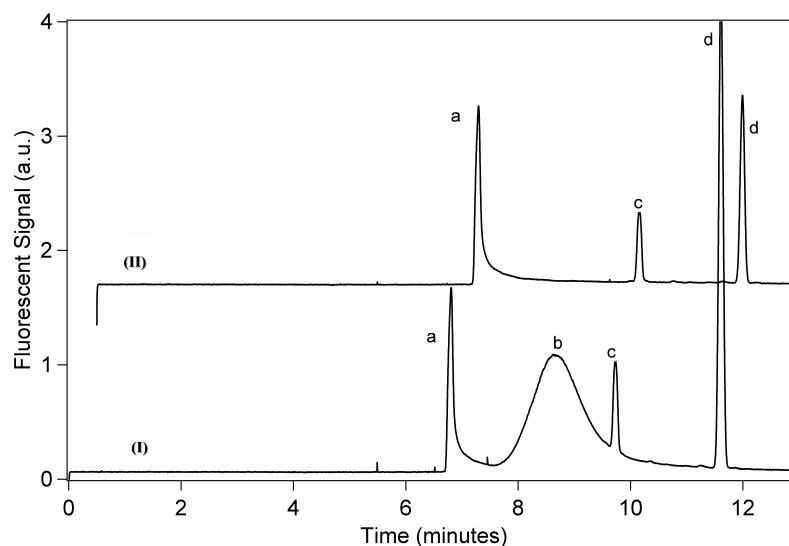


Figure 4.1 Separation of selected dyes: **(a)** $5.0 \mu\text{g ml}^{-1}$ alizarin, **(b)** $10 \mu\text{g ml}^{-1}$ purpurin **(c)** $10 \mu\text{g ml}^{-1}$ carmine, **(d)** $0.5 \mu\text{g ml}^{-1}$ morin, at 18 kV, 20 mM borate buffer (pH = 9.2) and 20 mM SDS. (I) fresh solution, (II) same solution after 1 hour. Adapted from Ref [1]. – Copyright: Elsevier

A second separation (II) of the same dyes solution was carried out approximately 60 minutes after the first separation and is also shown in Figure 4.1. In the second separation (II), the peaks for alizarin and carmine remained more or less unchanged. As opposed to the peaks for morin and purpurin, where both showed a decrease in peak size, particularly the peak for purpurin which decreased drastically to the point where it was essentially indistinguishable from the baseline. This result was unexpected since the alkaline conditions ($\text{pH} = 9.2$) of the borate buffer in these separations were similar to those by other researchers for the separation of purpurin and other anthraquinones [2-4]. It is interesting to note that in one study other researchers have reported challenges with the extraction of purpurin from madder when water was used as a solvent [4]. In another study difficulties with the separation of alizarin and purpurin in madder lake using CE [5] were also reported. Regarding flavonols, the photochemical degradation properties of selected flavonols were also investigated by other groups [6] particularly with quercetin and kaempferol [7]. Therefore, the problems observed in CE separation could be related to photochemical behaviors of the dyes.

This chapter pursues the CE separation issues regarding the peak shape and poor reproducibility of purpurin and some flavonols peaks. The objective of this research was to study the photochemical properties of these dyes. In Chapter 1 it was mentioned that purpurin and alizarin are very similar structurally with purpurin having one additional OH group on the aromatic ring, and flavonols within flavonoids are the least resistance to fading. Thus, UV-Vis was performed to evaluate the chemical structure changes of the fresh solution and 60 minute old of each dye at pH values: 7.0, 9.2 and 11.0. These pH values were chosen to have maximum and minimum criteria around the pH used in CE. The UV-Vis spectra for each class of dye were analysed and it showed

that purpurin was the most unstable dye among anthraquinones, as well as myricetin and quercetin among flavonols. In order to investigate the relative stability of these dyes the rate of photo-decomposition were studied by measuring the changes in fluorescence intensity over the time and plotting the natural log of fluorescence intensity of dyes as a function of time. The rate constant for photochemical fading of flavonols and purpurin at pH= 7.0, and 9.2, in light and dark conditions was measured. Thin layer chromatography, NMR, and GC-MS were also performed to identify the photodegraded products of purpurin. The degraded products of other dyes were not identified and this can be pursued in future studies.

In this study, the spectroscopic properties of purpurin and selected flavonols were investigated in an effort to explain the observations for their analysis using CE. It appeared that time and pH had a great impact on the changes in UV-Vis absorption and decline in fluorescence intensity of dyes. It could be concluded that, freshness and pH of dye solutions were extremely important for peak shape and reproducibility of peak height for these dyes.

4.2 Experimental

Separations were carried out on a laboratory-built CE instrument equipped with a post-column laser-induced fluorescence detection system [8]. The injection end of a 500 mm long, 10 μ m inner diameter fused silica capillary (Polymicro Technologies, Inc., Phoenix, AZ, USA) along with a platinum electrode connected to a high voltage supply (Spellman model CZE 2000, Hauppauge, NY, USA) was placed into a buffer-filled vessel. The applied voltage across the capillary were 18 and 22 kV. The detection end of the capillary, from which a 1 mm length of the external polyimide

coating was removed by flame, was inserted into a quartz sheath flow cuvette with a 250 by 250 μm inner bore (Hellma Ltd, Concord, ON, Canada). The system was grounded through the sheath flow buffer within the cuvette. A diode laser (Coherent, Inc., Santa Clara, CA, USA) with a 407 nm output (90 mW) was employed. Light from the laser was focused with a 6.3 X, N.A. 0.2 microscope objective (Melles Griot, Irvine, CA, USA) approximately 10 μm below the detection end of the capillary. Emission was collected at 90° using a 60 X, N.A. 0.7 microscope objective (Universe Kogaku, Inc., Oster Bay, NY, USA) passed through a 535AF45 optical filter (Omega Optical, Inc., Brattleboro, VT, USA) and a slit and then onto a photomultiplier tube (PMT, Hamamatsu model 1477, Middlesex, NJ, USA). The analog PMT signal was collected and digitized using a computer through a PCI-MIO-16XE I/O board utilizing LabVIEW® software (National Instruments™, Austin, TX, USA) at 10 Hz. The same board was used to control the electrophoresis voltage and PMT bias. Data was analyzed using IgorPro™ software (Instrutech, Port Washington, NY, USA). A Varian Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA, USA) was used to measure the spectra of purpurin. Measurements were carried out using a quartz cell with a 10 mm path length. The excitation and emission slits were set to 5 and 2.5 nm respectively. In addition to absorption spectra, emission spectra were also measured at a fixed excitation wavelength of 407 nm. The PMT was set at 1000 V for the samples in pH = 9.2 and 850 V for the samples in pH = 7.0. UV spectra were recorded on a Shimadzu UV 250 spectrophotometer (Shimadzu Co. Ltd., Kyoto, Japan).

Purpurin solutions of 5 $\mu\text{g ml}^{-1}$ at pH = 9.2 and 7.0; and flavonols solutions of 10 $\mu\text{g ml}^{-1}$ at pH = 9.2, 8.5 and 7.0 were exposed to a Solux lamp (3500 K Colour Temp, Colour Rendering Index: CRI 99, 10° spread; Solux, Rochester, NY, USA) for 1 minute to 7 hours with 1, 5, 10, 20

and 60 minutes intervals. For kinetics experiments, the light source was positioned 40 cm from the purpurin cuvette and 30 cm from the sample cuvette containing the flavonol dyes. The energy of this light source is 8.29 μW per lumen (380-315 nm). By positioning the dye samples 30 cm from the light source, the total lux was estimated to be 1.2×10^5 lumens/m².

Proton NMR spectra of fresh purpurin, photodegraded purpurin and phthalic acid were obtained using a Bruker 400 UltrashieldTM spectrometer (Bruker Biospin AG, Fallanden, Switzerland) in deuterated DMSO.

Mass spectrometry studies were carried out on a Hewlett- Packard 5890 gas chromatograph coupled with a Hewlett- Packard 5970 mass selective detector (GC-MSD) (Palo Alto, CA, USA). The samples were separated on a Hewlett- Packard 19091S-133 capillary column (30 m X 0.25 mm i.d., 0.5 μm thickness) type HP-5HS programmed from 70 to 275°C, a column flow of 1-2 ml minute⁻¹ at 70°C. The oven program was as follow: The initial temperature of the oven was 70°C for 2 minutes. Temperature was increased 7°C minute⁻¹ up to 160°C and held for 2 minutes and then it was increased 1°C minute⁻¹ up to 200°C and at the end of the program it was increased 7°C per minute up to 275°C and held for 5 minutes. The initial temperature of the MSD was 280°C. Mass spectra were recorded for a scan range of 50-550 m/z, with the standard threshold of 500 m/z. The solvent delay was defined as 4 minutes.

Thin layer chromatography (TLC), was performed on a 6.5 x 5.0 cm silica gel 60 F 254 TLC plate (EMD Chemicals Inc., Darmstadt, Germany). The samples were spotted by 25 μl micropipette (VWR international). The plate was developed in ethyl acetate and read in visible light and 254 nm UV light (Entela, USA).

4.3 Reagents

Dimethyl sulfoxide (DMSO), acetonitrile, sodium dodecyl sulfate (SDS) 99%, sodium hydroxide and purpurin and flavonol dyes (morin, Quercetin, myricetin) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Kaempferol was purchased from Alfa Aesar (Ward Hill, MA, USA). Diethyl ether was purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA), and sodium borate was obtained from Mallinckrodt (Phillipsburg, NJ, MO, USA). Potassium phthalic acid was from Mallinckrodt (Phillipsburg, NJ, USA) which was acidified with HCl and phthalic acid was extracted with diethyl ether. Ethyl acetate was from BDH (PA, USA), deuterated DMSO was purchased from CDN Isotopes (Quebec, Canada) and chloroform was obtained from Caledon Laboratories Ltd (Georgetown, ON, Canada). Fluorescein (Molecular Probes Inc., Eugene, OR, USA) was diluted and used for aligning the optics of the CE-LIF. Borate buffers used in this study also contained 20 mM SDS in Milli-Q water (18 M Ω ·cm) (EMD Millipore Corporation, Billerica, MA, USA). Borate buffers with varying pH were prepared using different ratios of boric acid and sodium tetraborate. Care was taken to maintain a constant ionic strength of the buffers at different pH to allow the comparison of migration times of different electropherograms. All solutions were filtered using a 0.45 μ m poly syringe filter. Standard solutions of dyes (1000 μ g ml⁻¹) were prepared by dissolving 10 mg of powder in 10 mL dimethyl sulfoxide (DMSO) and diluted as required to 0.5-10 μ g ml⁻¹ with buffer. All solutions were centrifuged prior to CE separation. Each new capillary was conditioned with sodium hydroxide solution (100 mM) for 30 minutes, and background electrolyte (BGE) for 20 minutes. Moreover, after each measurement the capillary was

rinsed with sodium hydroxide solution (100 mM) for 15 minutes and background electrolyte (BGE) for 10 minutes.

4.4 Discussion

Since the peak shape and reproducibility of peak height are affected by both time and pH, therefore UV-Vis spectrophotometry was performed on a fresh solution of each dye at different pH values, as well as on the same solutions after 60 minutes of exposure to light. By collecting the UV-Vis spectra and observing the changes in chemical structure of dyes at each pH value, the optimum pH for CE separation would be determined. The results would also show how freshness of sample solutions could affect the reproducibility of peaks in CE.

Then, the UV-Vis absorption properties of anthraquinones were studied. Purpurin was the most unstable dye belonging to this group and its photostability was investigated by performing fluorescence spectrophotometry. Next, the same pattern of study was applied to flavonoids: and the photostability of 4 dyes belonging to flavonols was investigated.

4.4.1 UV-Vis absorption properties of anthraquinones

The UV-Vis absorption of anthraquinone dyes were studied as a function of time and pH values of 7.0, 9.2 and 11.0. These pH values were chosen to have maximum and minimum criteria around the pH used in CE. For these experiments, 2.5 ml sealed quartz cuvettes containing $10\text{ }\mu\text{g ml}^{-1}$ of each dye were prepared in 20 mM borate buffer and 20 mM SDS. The cuvette was sealed to minimize evaporation of solvent which allowed the UV-Vis spectra to be acquired after 60 minutes. The results of UV-Vis absorption for anthraquinones are presented in **Figure 4.2**.

As it can be seen all of dyes have 3 absorption bands corresponding to the chemical structure of groups in the anthraquinones. The absorption observed in UV region (240-270 nm) is due to electron transfer of benzenoid ring and one ketone group in the benzoquinone ring. However, the ketone groups cause 2 other absorption bands, between 285-345 nm and 345-654 nm, where the first band is due to the electron transfer and the second one is due to the local excitation of ketone functional groups [9].

The absorption spectra of alizarin at pH= 7.0 and 9.2 showed the same shape and intensity with the maximum absorption around 450 nm. By increasing the pH to 11.0 the spectrum shifted to the red ($\lambda_{\text{max}} = 500 \text{ nm}$) **Figure 4.2-a**. According to literature, alizarin has 2 ionizable enolic functional groups with $\text{pK}_{\text{a1}} = 6.6$ and $\text{pK}_{\text{a2}} = 12.4$ (**Figure 1.1**) that can be ionized in strong alkaline conditions [10]. A net isosbestic point at 500 nm is assigned to the mono-anion form of alizarin. The absorption changes observed after 1 hour was approximately the same as results observed initially ($t = 0 \text{ minute}$).

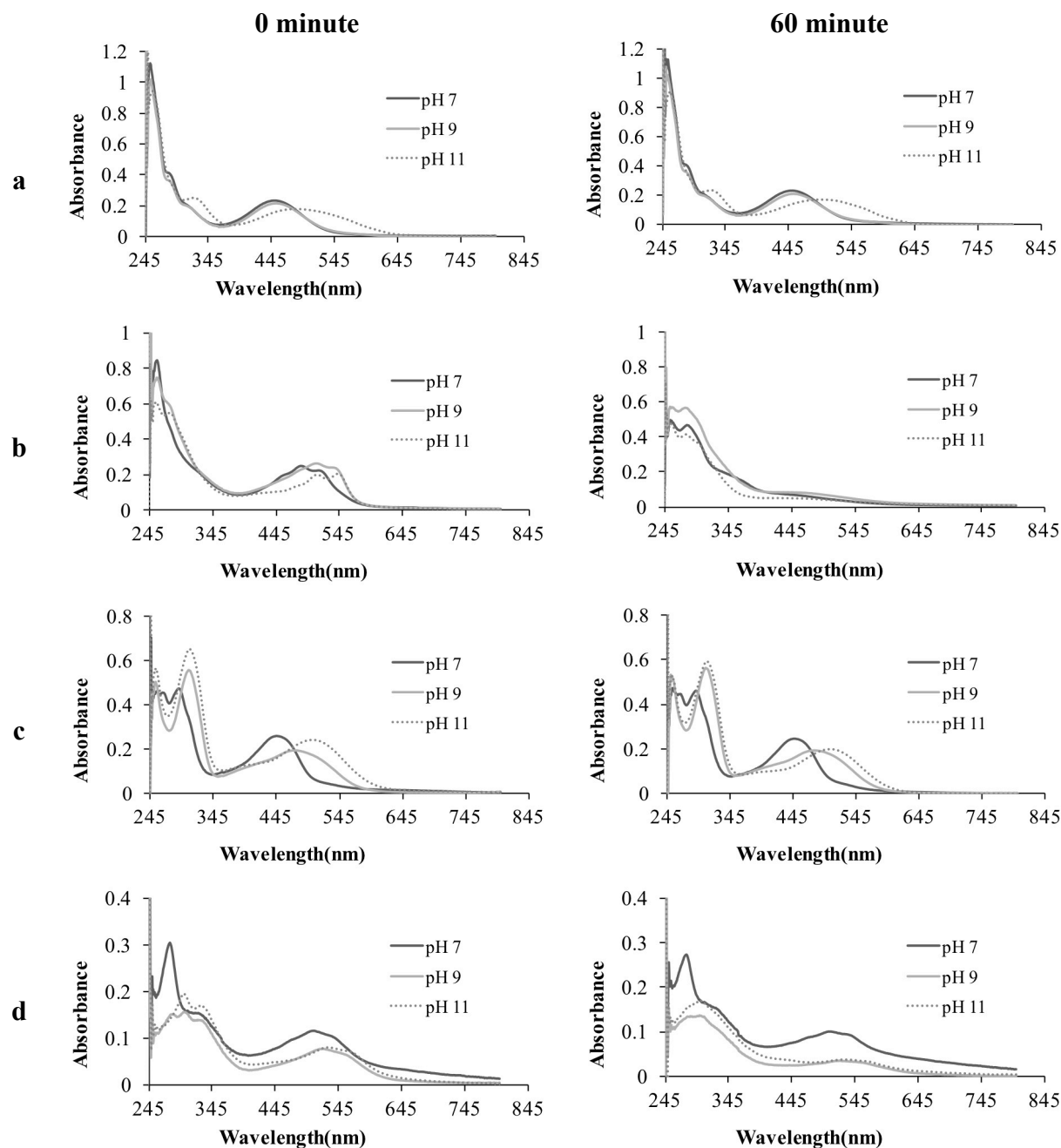


Figure 4.2 The absorption spectra of anthraquinones at pH= 7.0, 9.2 and 11.0: **a:** alizarin, **b:** purpurin, **c:** emodin, and **d:** carmine.

It indicates that in this time frame the mono-anion form of alizarin was stable and did not appear to undergo decomposition.

The absorption spectra of purpurin at pH= 7.0, 9.2 and 11.0 are depicted in **Figure 4.2-b**. Purpurin has $pK_{a1} = 4.7$ and $pK_{a2} = 9.5$ [10], therefore, it can be concluded that the spectrum belonging to pH= 7.0 could be assigned to mono-anion form of purpurin with $\lambda_{max} = 512$ nm.

By increasing the pH (pH= 9.2), the absorption spectrum shifted to longer wavelength and caused a slight increase in absorbance (the molar absorptivity of dyes is given in Appendix A, **Table A.4.1**), at pH=11.0, a vibronically structure spectrum appeared with 2 absorption peaks at 510 nm and 541 nm with lower intensity. Isosbestic points appeared at 440 nm and 550 nm for the neutral-mono-anion and mono-anion and di-anion couples, respectively. **Figure 4.2-b** shows that after 1 hour the absorption in the visible range (400- 600 nm) in all pH conditions declined remarkably. It indicates that mono and di-anion forms of purpurin were not stable and decomposition occurred.

The absorption spectra of emodin are presented in **Figure 4.2-c**. By increasing the pH, the maximum absorption shifted to longer wavelength. By considering the acid dissociation of emodin $pK_{a1} = 5.7$ and $pK_{a2} = 7.94$ [11] it can be concluded that the spectrum at pH =7.0 with $\lambda_{max} = 447$ nm is due to mono-anion form of emodin. A net isosbestic point was observed at 471 nm for mono-anion and di-anion couple. The absorption spectra of emodin after 1 hour were approximately the same as for minute zero. It can be due to the stability of mono and di-anion forms of emodin during this time frame, and they might have shown a decline over a longer period of time. Al-Nuri et al. studied the effect of pH on absorption spectra of emodin and it was observed that maximum absorption at 520 nm at pH=9 is due to the dissociation of di-anion to tri-anion and by increasing the pH the λ_{max} did not change [12].

Figure 4.2-d. shows the absorption spectra of carmine under alkaline pH values, with the maximum absorption around 520 nm. By increasing the pH to 11.0 the spectrum revealed a red shift ($\lambda_{\text{max}} = 529$ nm). Carmine has one carboxylic group with $\text{pK}_{\text{a0}} = 2.9$ and 3 ionizable enolic functional groups at positions C-6, C-3 and C-8 with $\text{pK}_{\text{a1}} = 5.62$, $\text{pK}_{\text{a2}} = 8.7$ and $\text{pK}_{\text{a3}} = 12.2$, respectively. The enolic groups at C-5 and C-8 are less acidic due to the reducing of hydrogen mobility through hydrogen bonding with carbonyl groups, the tetra- anion would be formed at $\text{pH} > 12$ [13].

4.4.2 Photochemistry of purpurin

The results from UV-Vis absorption showed that purpurin was the most unstable dye among anthraquinones. To investigate the relative stability of purpurin, fluorescence spectrophotometry was also performed. The rate of photo-decomposition was determined by measuring the decrease in the fluorescence intensity over time. The reasons why fluorometry was preferred over spectroscopy for this study were high sensitivity and low detection limits as well as using solutions with lower concentrations. For these experiments, the fluorescence of purpurin was measured using a bench top fluorimeter with an excitation wavelength of 407 nm (5.0 nm slit). Addition for these experiments, buffered solutions (20 mM borate and 20 mM SDS) containing purpurin were exposed to light from Solux bulbs. The spectral output of the light source used for this experiment was Gaussian shaped with a maximum at 600 nm. The quantity of energy hitting the sample was 15 μW when placed at 200 mm from the light source. These light sources were chosen because they produce a continuum of light in the visible spectrum. **Figure 4.3 (A-D)** shows the fluorescence behavior of a 10 $\mu\text{g ml}^{-1}$ solution of purpurin under light (A, C) and dark (B, D) conditions at $\text{pH} = 7.0$ and $\text{pH} = 9.2$. **Figure 4.3-A** and **4.3-B** show the effect of visible light and darkness on

the fluorescence of $10 \mu\text{g ml}^{-1}$ purpurin at $\text{pH} = 9.2$ respectively. Under these conditions, it was observed that purpurin was fading after a few minutes, even with the unaided eye. This property was not obvious visually when the dyes were present in a mixture, especially if other dyes are providing colour to the solution (Figure. 4.1). The photochemical change in purpurin buffered at $\text{pH} = 9.2$ is quite significant and after 8-10 minutes the solution became faded completely. For comparison, a similar experiment was carried out with a $10 \mu\text{g ml}^{-1}$ purpurin solution buffered at $\text{pH} = 9.2$ except the cuvette was shielded from visible light and the results of this experiment are shown in **Figure 4.3-B**.

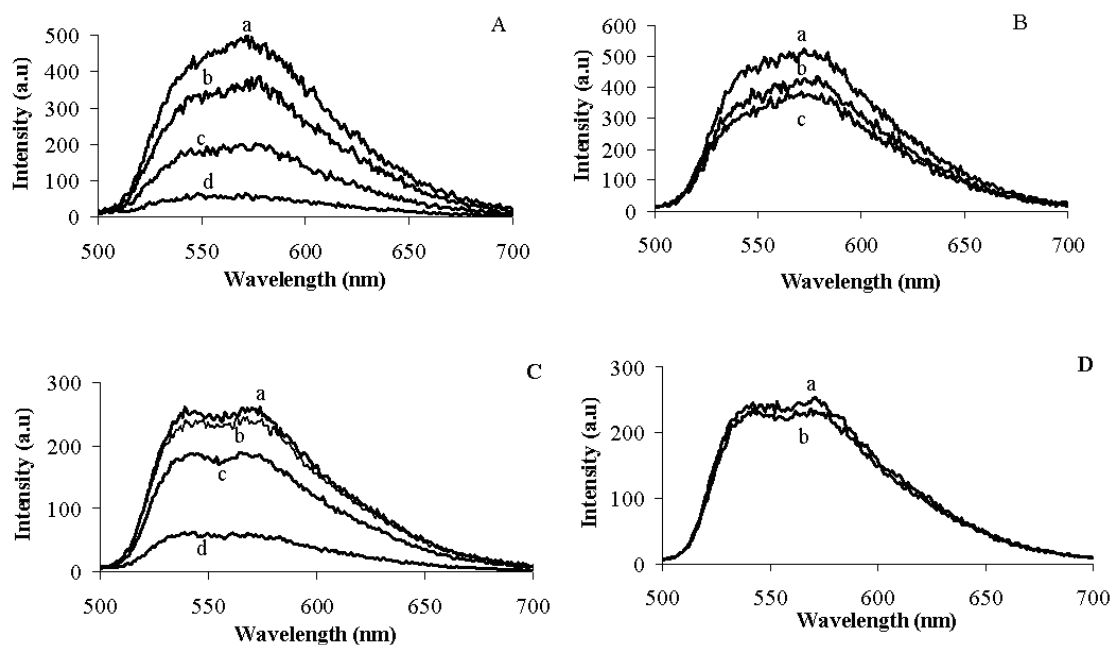


Figure 4.3 Changes in the fluorescence signal of purpurin over time under light and dark conditions at different times **(A)** light, $\text{pH} = 9.2$ (a = 0, b = 1, c = 3, d = 8 minutes); **(B)** dark, $\text{pH} = 9.2$ (a = 0, b = 20, c = 264 minutes) dark; **(C)** light, $\text{pH} = 7.0$ (a = 0, b = 10, c = 90, d = 480 minutes); **(D)** dark, $\text{pH} = 7.0$ (a = 0, b = 953 minutes). Adapted from Ref [1]. – Copyright: Elsevier

Although some fading was observed under these conditions, the rate was significantly slower in

the dark. This appeared to indicate that a photochemical reaction was the cause of the fading of purpurin.

In order to understand the role of pH in this reaction, a similar experiment was also carried out at pH = 7.0 under light and dark conditions. **Figure 4.3-C** shows the fading of a $10 \mu\text{g ml}^{-1}$ purpurin solution when exposed to visible light. Under this condition, fading of purpurin was still occurring, however the rate of reaction was significantly slower. For comparison, the fluorescence of an identical purpurin solution was also measured under dark conditions (**Figure 4.3-D**) and under the same pH conditions. Under these conditions, the purpurin solution was quite stable, photo-chemically, even after a relatively long period of storage.

In order to assess the relative stability quantitatively, kinetics experiments were carried out to determine reaction order and rate constants under different pH conditions. Part of the motivation in assessing the order for this photochemical reaction was to observe whether other reactions were occurring under alkaline conditions (pH = 9.2). In order to verify if the decomposition of purpurin at each pH value follows the 1st order reaction, the natural log of fluorescence intensity of purpurin was plotted with respect to time under different pH conditions. Since all graphs had a linear profile with a negative slope, the reactions must be 1st order reactions following the 1st order rate equation *Eq. 1*.

$$\ln[\text{Fluorescence}] = -kt + \ln [\text{Fluorescence}]_0 \quad \text{Eq. 1}$$

where:

$\ln[\text{Fluorescence}]$ = natural log of fluorescence intensity of purpurin at time t

k = 1st order rate constant s^{-1} ,

$\ln [\text{Fluorescence}]_0$ = natural log of fluorescence intensity of purpurin at time 0

Figure 4.4 shows the natural log of fluorescence plotted with respect to time under different pH conditions. For these experiments the decrease in fluorescence of purpurin followed 1st order kinetics with a high degree of linearity ($r^2 = 0.99$ or higher) under all pH conditions. For comparison, when the fluorescence data were also plotted using 2nd order kinetics, a linear plot was not achieved. Based on this it can be concluded that a 1st order reaction mechanism can be used to describe the photochemical fading of purpurin.

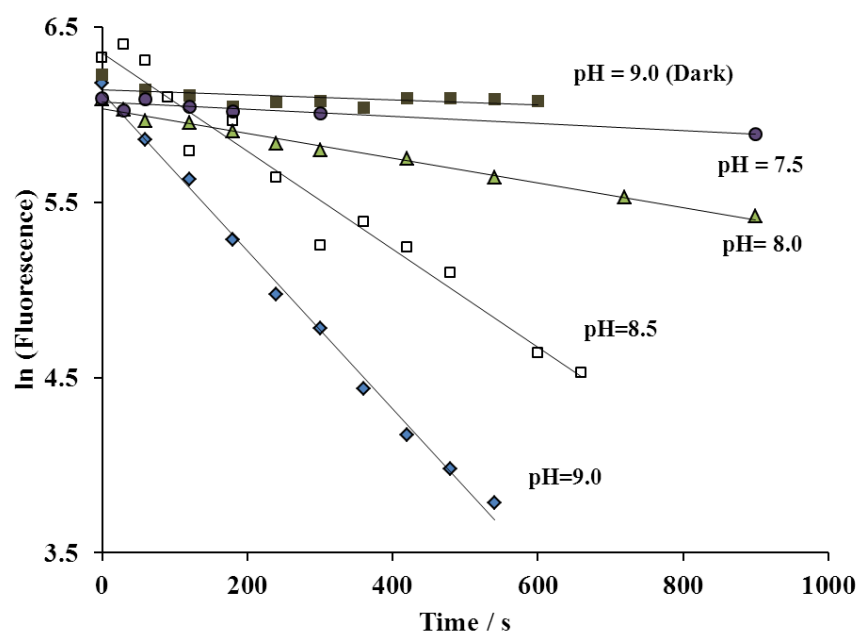


Figure 4.4 The change in fluorescence of purpurin after exposure to visible light under different pH conditions. Adapted from Ref [1]. – Copyright: Elsevier

Using the data from **Figure 4.4** 1st order rate constants were calculated for this reaction under different pH conditions and the results are summarized in **Table 4.1**.

For comparison, rate constants for the fading of purpurin were also calculated under dark conditions. The half-life data in **Table 4.1** demonstrates that the stability of a 10 $\mu\text{g mL}^{-1}$ solution can be doubled simply by decreasing the pH from 9.2 to 8.5. Decreasing the pH of a buffer solution

containing purpurin can certainly have noticeable beneficial effects on the peak shapes of most anthraquinone dyes depending on their respective pK_a value. Unfortunately, decreasing the pH can have serious consequences on the separations of dye mixtures, especially if their respective pK_a values are similar.

Table 4.1 Rate constants for the decrease in fluorescence of purpurin under light and dark conditions Adapted from Ref [1]. – Copyright: Elsevier

pH	Light		Dark	
	$k(s^{-1})$	Half-Life (s)	$k(s^{-1})$	Half-Life (s)
9.0	4.5×10^{-3}	158	9×10^{-6}	7.70×10^4
8.5	2.1×10^{-3}	330	9×10^{-6}	7.70×10^4
8.0	0.6×10^{-3}	1.16×10^3	8×10^{-6}	8.66×10^4
7.5	2.0×10^{-4}	3.47×10^3	1×10^{-6}	6.93×10^5
7.0	5.0×10^{-5}	13.9×10^3	1×10^{-6}	6.93×10^5
6.5	8.0×10^{-6}	86.6×10^3	0.9×10^{-6}	6.93×10^5

Experiments were also carried out to determine the identity of the degradation products. The goal of this part of the research was not to develop a reaction mechanism, rather it was to confirm the decrease in fluorescence was consistent with the expected decomposition products. Spectrophotometry revealed that the decrease in fluorescence was consistent with the formation of a decomposition product. **Figure 4.5** shows the change in the UV-Vis absorption spectra of purpurin in borate buffer at pH = 9.2 over time.

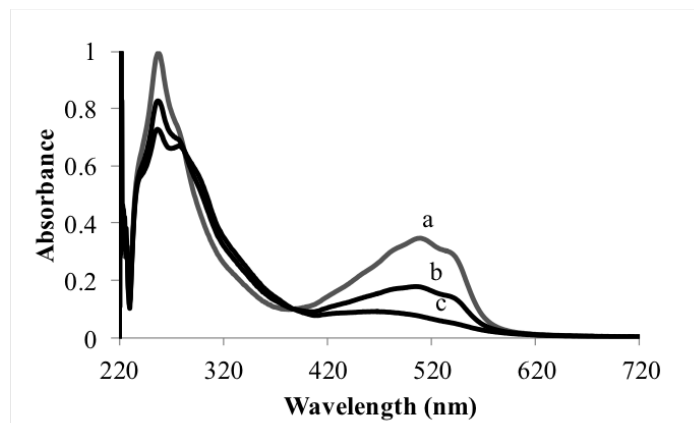


Figure 4.5 Changes in the UV-Vis absorption spectra of purpurin over time under light condition at pH = 9.2 at different times (**a** = 0, **b** = 20, **c** = 60 minutes). Adapted from Ref [1]. – Copyright: Elsevier

Previous research has speculated the formation of phthalic acid and salicylic acid as a result of decomposition of purpurin, [14] [15] however structural information was not provided. **Figure 4.6** shows a UV-Vis spectrum of phthalic acid in borate buffer at pH=9.2. The small peak which appeared in faded purpurin at 280 nm appears to be consistent with phthalate, however other approaches were required to reveal the decomposition product with a reasonable amount of certainty.

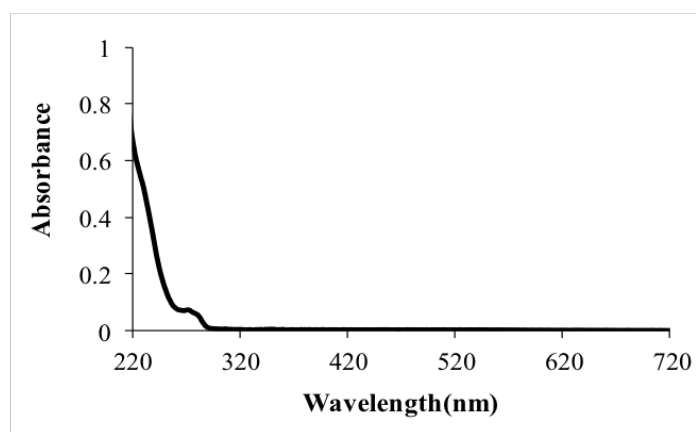


Figure 4.6 Absorption signal of phthalic acid at pH = 9.2. Adapted from Ref [1]. – Copyright: Elsevier

Thin layer chromatography (TLC) was also employed to determine if other decomposition products were formed from the photodecomposition of purpurin. There were 2 faded purpurin samples, one was faded in borate buffer and the other faded in deuterated chloroform. $1000 \mu\text{g ml}^{-1}$ standard solutions of oxalic acid, salicylic acid, potassium phthalate acid (KHP), and $200 \mu\text{g ml}^{-1}$ standard solution of phthalic acid in ethyl acetate were prepared. The standard solutions and 2 faded purpurin samples were spotted with a $25 \mu\text{l}$ micropipette onto a TLC plate. The results of TLC separations (**Figure 4.7**) indicated the formation of 2 decomposition products from the faded purpurin and the mobility of these species were similar to that of salicylic acid (2) and potassium phthalate acid (3).

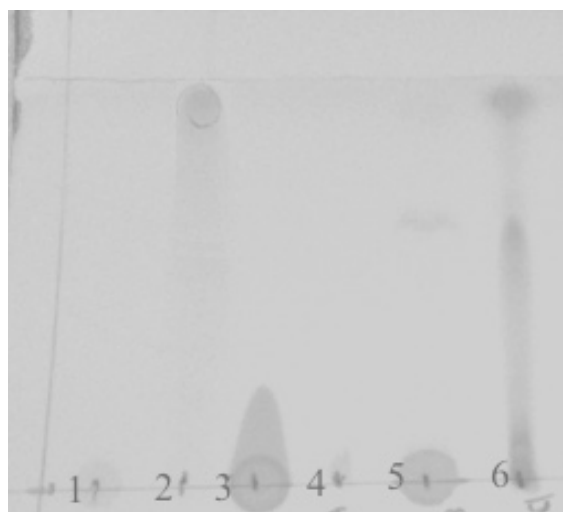


Figure 4.7 Thin-layer chromatogram of: 1= oxalic acid, 2= salicylic acid, 3= potassium phthalate acid (KHP), 4= phthalic acid, 5= faded purpurin dissolved in borate buffer, 6= faded purpurin dissolved in CDCl_3 .

Proton (^1H) NMR studies were carried out on purpurin using deuterated DMSO and CHCl_3 as solvents. The ^1H NMR of fresh purpurin in DMSO-d_6 (**Figure A.4.1**) showed a singlet at δ 6.68 ppm (1H, s, H-3), multiples at δ 7.91-7.95 ppm (2H, m, H-6 and H-7), multiples at δ 8.21-8.23 ppm (2H, m, H-5 and H-8), and singlets for the phenolic hydroxyl groups (OH-1, OH-2, OH-4)

at δ 11.63, 13.12 and 13.39 ppm. The broad peak at δ 11.63 belongs to OH-2 which indicates weak intra-molecular H-bonding. The ^1H NMR of faded purpurin in DMSO-d_6 (**Figure A.4.2**) showed signals belonging to purpurin as well as faded products. The ^1H NMR spectra of both purpurin and faded purpurin were found to be similar except for the 2 multiples at δ 7.57-7.63 ppm (2H, m, H-4 and H-5) and δ 7.77-7.78 ppm (2H, m, H-3 and H-6) as well as a singlet for the phenolic hydroxyl group OH-3. The ^1H NMR of phthalic acid in DMSO-d_6 (**Figure A.4.3**) also gave a pair of multiples at δ 7.57-7.60 ppm (2H, m, H-4 and H-5) and δ 7.65-7.68 ppm (2H, m, H-3 and H-6), the broad peak at δ 13.05 ppm is assigned to OH in the 2 carboxylic acid groups. These signals are consistent with those detected in faded purpurin. The NMR data for the faded purpurin was helpful in that it provided structural information that was consistent with phthalic acid. Unfortunately, the low sensitivity of ^1H -NMR did not permit the identification of other decomposition species.

Gas chromatography with mass spectrometry (GC-MS) was also used to detect the decomposition compounds in a $10\text{ }\mu\text{g ml}^{-1}$ solution of purpurin after exposure to light. For this experiment CHCl_3 was used as a solvent and the GC-MS analysis of the faded purpurin solution identified as phthalic anhydride (phthalate ion) as the most significant species. When compared to the phthalic anhydride, other species such as salicylic acid (salicylate ion) and oxalic acid (oxalate ion) were not detected in significant quantities **Figure 4.8** shows their chemical structures.

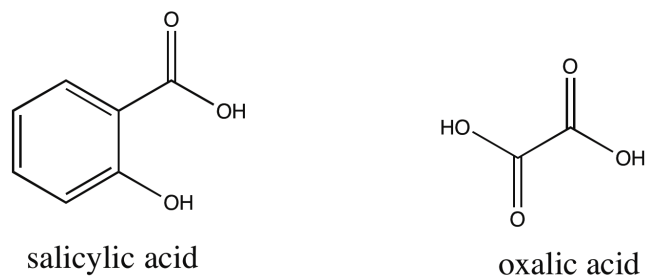


Figure 4.8 The chemical structures of salicylic acid and oxalic acid

Ahn *et al.* studied the mechanism of aerobic degradation pathway for alizarin and they believed the degradation products of other anthraquinones such as purpurin would be the same as alizarin. **Figure 4.9** shows the mechanism and the degraded products introduced by Ahn et al [16].

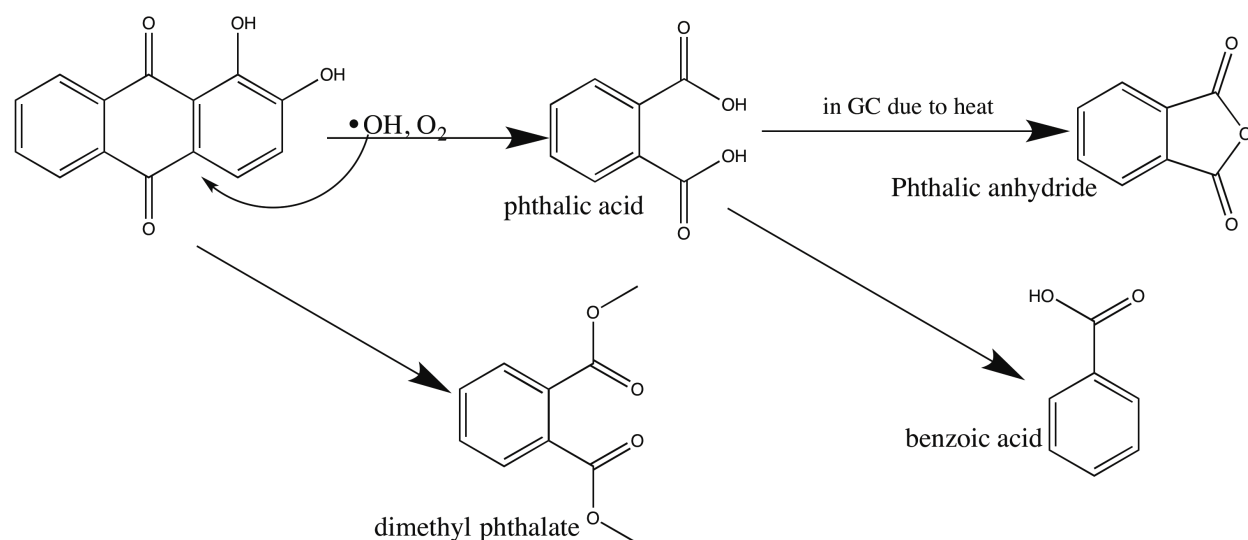


Figure 4.9 The mechanism of aerobic degradation pathway for alizarin and degraded products

The gas chromatogram and mass spectra of faded purpurin and phthalic acid are provided in appendix A: **Figures A.4.4- A.4.6**. Based on the kinetics experiments it was clear that pH could play an important role in improving the separation properties of purpurin. As shown in Chapter 3 the peak shape and peak reproducibility of purpurin was affected drastically by slight changes in

pH. Regarding flavonols under alkaline conditions the reproducibility in peak height and peak shape was poor for morin and quercetin. Therefore, it was decided to study the UV-Vis absorption properties as well as the photochemical degradation properties of selected flavonols by following the same procedure applied for purpurin. Except for the concentration of dyes and the distance from the light source were different: $10\text{ }\mu\text{g ml}^{-1}$ vs $5\text{ }\mu\text{g ml}^{-1}$, and 20 vs 30 cm.

4.4.3 UV-Vis absorption properties of flavonoids

The flavonols used for this study were: kaempferol, quercetin, morin and myricetin. As mentioned in introduction under alkaline conditions ($\text{pH} > 9$) peak height of dye morin was also observed to decrease noticeably with successive separations over periods of time that exceeded 1 hour. The observed decrease in peak height ultimately resulted in a significant decrease in the sensitivity for these types of measurements over time. In order to evaluate the extent of this problem with flavonol dyes, UV-Vis spectrophotometry was employed to observe changes in the spectral properties of flavonoids which would be indicative of changes in the flavonol dye structure. The UV-Vis absorption of flavonoids were studied employing the same procedure used for anthraquinones.

The close consideration of chemical structure of flavonoids **Figure 4.10** shows that they are made of 2 major systems: benzoyl and cinnamoyl consisted of A-ring and half of ring C; B-ring and other half of ring C, respectively. UV spectra of flavonoids are consisted of 2 major bands known as band I and II, which are located at 300-380 nm and 240-280 nm, respectively. Band I and II are considered to be associated with absorption involving B-ring and A-ring, respectively [17].

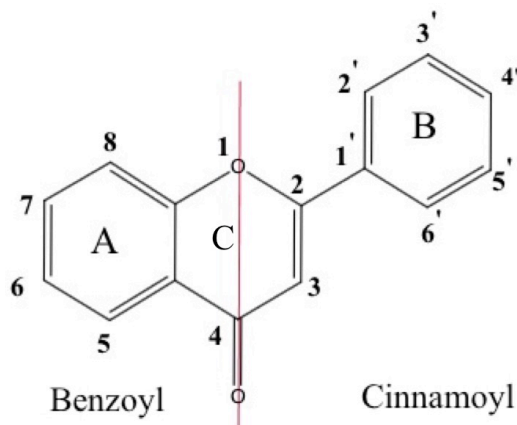


Figure 4.10 The benzoyl and cinnamoyl systems in flavonoids

The UV spectra of oxygenated A-ring flavonoids have more intense peaks in band II. Also, by introduction hydroxyl groups in B-ring in the same system (oxygenated A-ring flavonoids), show more intense peaks in band I with red shift. The position of band I helps to distinguish between flavones and flavonols. Where band I of flavones and flavonols occur between 304-350 nm and 352-385 nm, respectively. It was reported in the case of B-ring ortho-dihydroxy flavones in the presence of sodium acetate /boric acid, band I had a consistent 12- 30 nm red shift, which is the evident of formation of the borate complex (**Figure 4.11**). The red shifts for luteolin, lu-7-*O*-glu, quercetin and myricetin are reported as: 21 nm, 24 nm, 17 nm and 18 nm, respectively [17].

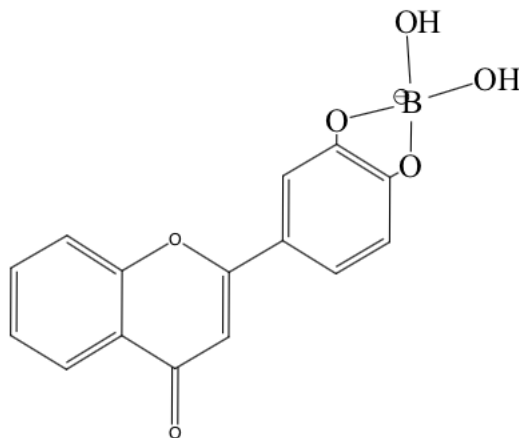


Figure 4.11 The borate complex with ortho- dihydroxy flavones

The absorption maxima of apigenin and luteolin in methanol are reported at 337 nm and 347 nm, respectively [18]. The UV absorption spectra of luteolin and apigenin at different pH values at zero minute are shown in **Figure 4.12**.

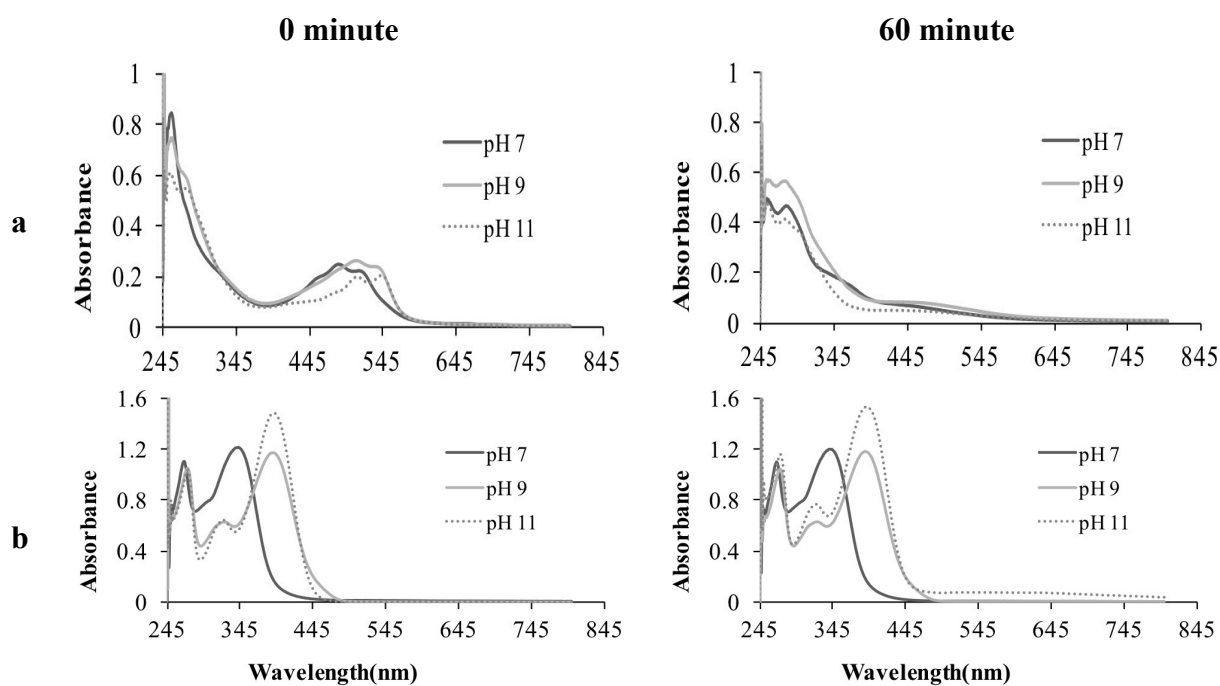


Figure 4.12 The absorption spectra of flavones at pH= 7.0, 9.2 and 11: **a:** luteolin, **b:** apigenin.

The changes of UV absorption of luteolin and apigenin after 1 hour also were investigated and the results are presented in **Figure 4.12-a** and **4.12-b**. Also, the $\lambda_{\max}$ of band I of flavonoids at different pH values are summarized in **Table A.4.1**. It can be seen that at pH= 7.0, the $\lambda_{\max}$ for apigenin and luteolin occurred at 342 nm and 372 nm, respectively. These findings are in agreement with those of reported for apigenin and luteolin in the solution of sodium acetate and boric acid were: 338 nm and 370 nm, respectively [17]. This is an evident that luteolin with the B-ring ortho-dihydroxy structure, has formed the borate complex. According to Markham *et al.* the UV absorption maxima of 7-*O*-glycosylated apigenin and luteolin located at longer wavelength: 340 nm and 372 nm, respectively [16].

It is worth noting, the UV spectra of methylated or glycosylated flavonols at position 3 (the difference between flavones and flavonols is the hydroxyl group at position 3), display blue shift and their band I occur between 324-325 nm, which indicate that the band has shifted toward the flavones band I. The Results of UV-Vis absorption for flavonols are presented in **Figure 4.13**

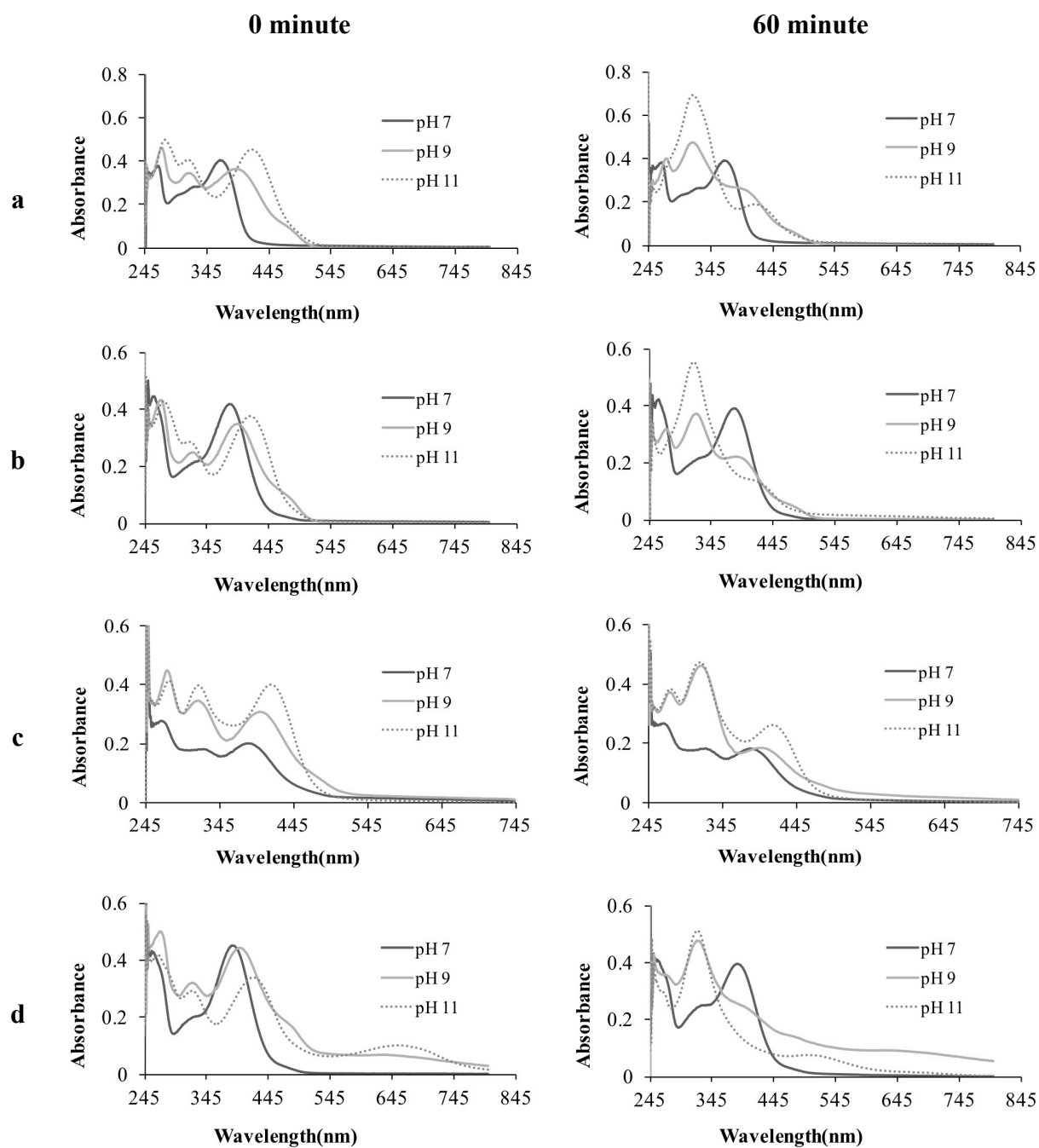


Figure 4.13 The absorption spectra of flavones at pH=7.0, 9.2 and 11: **a**: kaempferol, **b**: quercetin, **c**: morin, **d**: myricetin.

Table 4.2 shows the absorption maxima of flavonoids reported by Markham *et al.* [16] in methanol and borate solution as well as our findings in borate buffer at pH values of 7.0, 9.0 and 11.0. It can be concluded that in the case of quercetin and myricetin with the B-ring ortho-dihydroxy structure, the borate complex form at pH= 7.0 and 9.0, respectively.

Table 4.2 The absorption maxima of flavonoids in methanol and borate buffer [17] and my data

Dye	$\lambda_{\max}$ (nm) in methanol	$\lambda_{\max}$ (nm) in borate	$\lambda_{\max}$ (nm) my data		
			pH= 7.0	pH= 9.0	pH= 11.0
kaempferol	367	372	368	390	419
quercetin	370	386	382	395	414
morin	370	374	381	398	415
myricetin	374	392	388	397	419

By comparing the changes of molar absorptivity of flavonoids at all pH values after one hour (**Table A.4.1**) it can be concluded that luteolin, apigenin at all pH values and kaempferol (only at pH=7.0) were the one flavonol dye that showed slight changes as opposed to quercetin, morin and myricetin, where the percentage of changes of their molar absorptivity increased with increasing of pH. The percentage decrease of molar absorptivity for myricetin, quercetin and morin at pH= 7.0, 9.2 and 11.0 after 60 minutes showed the following trend: myricetin > quercetin > morin. Myricetin showed the highest percentage decrease: 21.60% (pH=7.0), 79.08 % (pH=9.2), and at pH=11.0, after 60 minutes the absorption could not be detected. The decrease of molar absorptivity (ϵ) for quercetin at pH values of 7.0, 9.2 and 11.0 after 60 minutes were :11.14%, 48.70% and 90.26%, respectively. (It shows that the most unstable flavonoid is myricetin).

Structural change that occurs in these flavonol dyes can be explained as follows: the 3 absorption peaks in the non-degraded dyes are evident at between 280 and 440 nm. These absorption

peaks occur at approximately the same wavelengths for each dye in the UV and the shorter visible regions of the electromagnetic spectrum. The wavelengths of these absorption peaks can be observed at 284 nm, 324 nm and 420 nm. After 60 minutes, the peaks at 284 nm and 450 nm decreased significantly in magnitude and the peak at 324 nm appeared to increase. As these dyes were dissolved in an alkaline solution, it would seem logical that the C3-OH group on the 'B' ring of the dye structure was deprotonated [19] [20]. Under alkaline conditions, de-protonation is thought to lead to oxidation and fragmentation resulting in the formation of hydroxyl benzoic acids. This is observed by the rather dramatic increase in the peak at 310-324 nm in the absorption spectrum [21]. **Figure 4.14** shows the fragmentation mechanism as well as degraded products of quercetin [21].

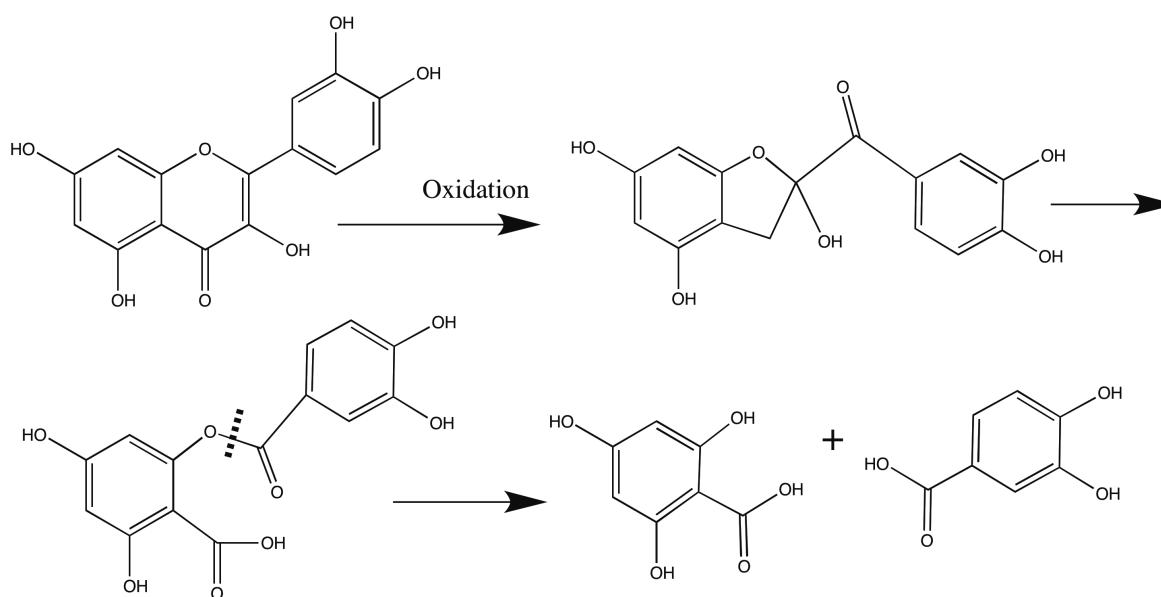


Figure 4.14 The fragmentation pathway of quercetin resulted in hydroxyl benzoic acids.

4.4.4 Photochemistry of flavonols

Fluorescence spectrophotometry was used to investigate the chemical properties associated with the photochemical decomposition flavonols. For these experiments the fluorescence of each dye was measured following a similar procedure used for purpurin, except that the concentration of myricetin was $10\ \mu\text{g ml}^{-1}$ and the distance between cuvette and light source was 30 cm. In following the detail of this study was enlightened by presenting myricetin as the representative of flavonols which was the most unstable dye in alkaline condition. **Figure 4.15** shows the change in fluorescence spectra that was observed for $10\ \mu\text{g ml}^{-1}$ myricetin which was prepared in 20 mM borate buffer at pH = 9.2.

In this experiment, the myricetin solution was placed in a quartz cuvette and exposed to visible light from a Solux bulb. After 1 hour of light exposure, a decrease in fluorescence of the myricetin could be detected. After 3 hours, the fluorescence signals were approximately 25% lower (650 counts) than the peak height of the fluorescence signals that were acquired initially (880 counts, $t = 0$). It is worth noting that the same myricetin solution which was kept under dark conditions, had relatively little change in fluorescence intensity. In this case, it is possible that the small decrease in fluorescence under dark conditions can partially be attributed to unavoidable light exposure when taking the fluorescence measurement. However, it may also indicate more likely some de-protonation and possibly oxidation of the dye solution under alkaline conditions [7].

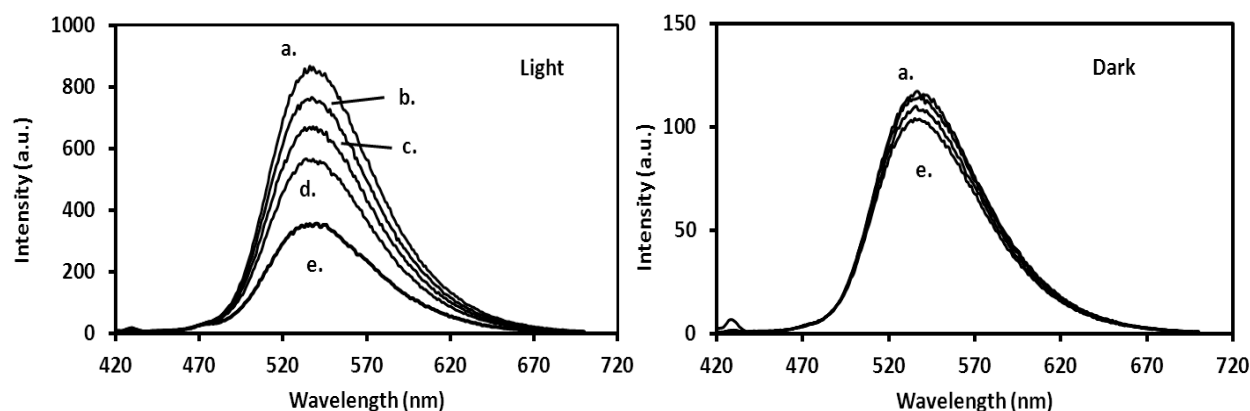


Figure 4.15 Decrease in fluorescence of myricetin ($10 \mu\text{g ml}^{-1}$) in 20 mM borate buffer ($\text{pH} = 9.2$) over time: **a** = 0, **b** = 120, **c** = 180, **d** = 240 and **e** = 360 minutes under light conditions and dark conditions. Excitation wavelength = 407 nm. Adapted from Ref. [22] – Copyright: Taylor and Francis

Nevertheless, the exposure of a dilute solution of myricetin to light from a visible light source (380-750) indicates the extent of photo-degradation of this dye over time. Although, not shown, similar results were also observed for $10 \mu\text{g ml}^{-1}$ solutions of kaempferol, quercetin, and morin under these conditions.

In order to compare the relative stability of these dye solutions, the rate of photo-decomposition was determined by measuring the decrease in the fluorescence over time. For this experiment, each dye solution was placed in a quartz cuvette prior to exposure from the light source. The magnitude of the fluorescence intensity was measured after specific time intervals for dye solutions that were exposed to light as well as dyes that were kept under dark conditions. To monitor instrument drift a dilute solution of fluorescein ($10 \mu\text{g ml}^{-1}$) was also prepared and its fluorescence was measured at the same time as the dyes. The natural log of the fluorescence peak height was then plotted with respect to time. **Figure 4.16** shows the decrease in fluorescence signals of $10 \mu\text{g ml}^{-1}$ solutions of kaempferol, quercetin, morin and myricetin, under both light and dark conditions.

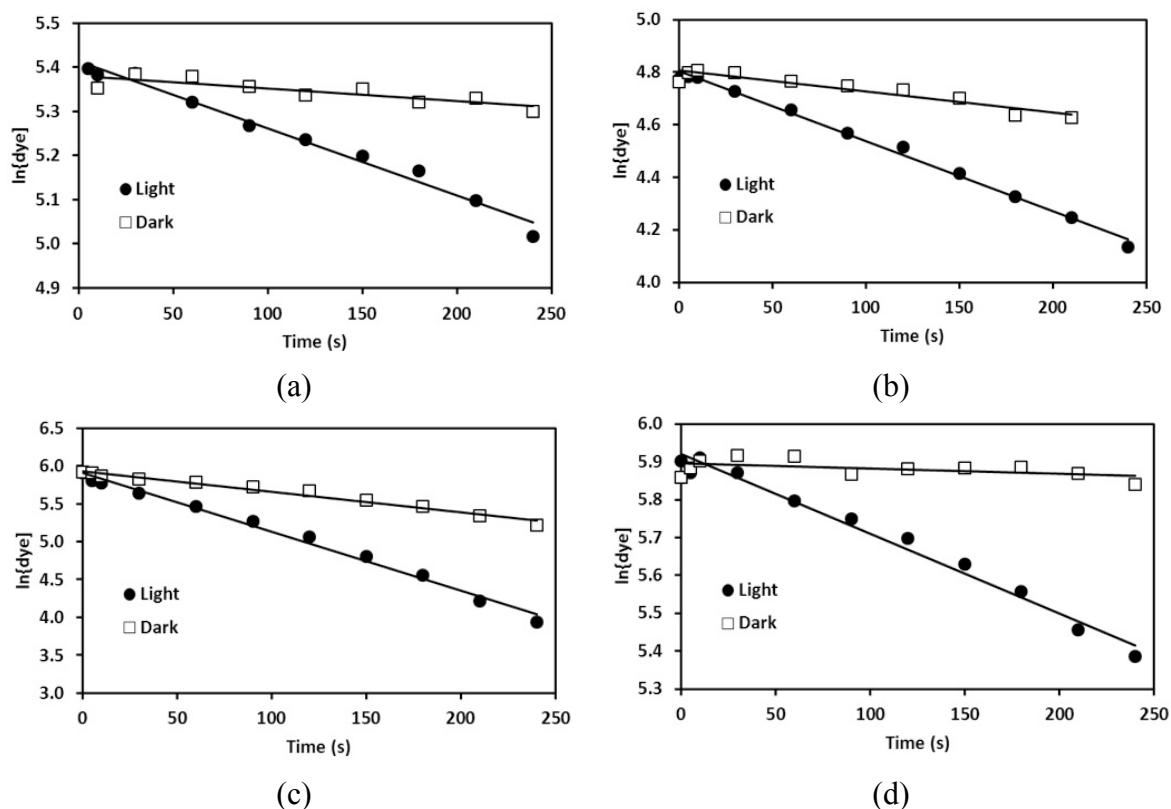


Figure 4.16 Plot of $\ln$ (fluorescence) of: **a:** kaempferol, **b:** quercetin, **c:** myricetin and **d:** morin with respect to time. Each dye ($10 \mu\text{g ml}^{-1}$) was dissolved in 20 mM borate buffer (pH = 9.2). Adapted from Ref. [22] – Copyright: Taylor and Francis

These plots revealed that the decrease in fluorescence of each dye appeared to follow 1st order kinetics. The significant decrease in fluorescence under dark conditions would strongly suggest that another degradation process (oxidation) was likely taking place as well, specially in case of quercetin **Figure 4.16-b** [7]. Second order plots were also constructed using the same fluorescence data for these dyes, however none of these reactions demonstrated a linear relationship between $[\text{dye}]^{-1}$ and time. Although degradation by oxidation must be occurring, under these conditions only the 1st order kinetics of the photochemical decomposition was being observed. In order to compare the rates of photochemical decomposition, 1st order rates constants, k of each dye were calculated at pH = 7.0, 9.2 and 11.0 and the results are presented in **Table 4.3**.

Table 4.3 First-order rate constants calculated from fluorescence spectrum data in 20 mM borate buffer at pH 7.0, 9.2, and 11.0 under light and dark conditions. Reproduced from Ref. [22] – Copyright: Taylor and Francis

pH	Light				Dark			
	Rate constant (s ⁻¹)				Rate constant (s ⁻¹)			
	kaempferol	quercetin	morin	myricetin	kaempferol	quercetin	morin	myricetin
7.0	0.018	0.024	0.012	0.06	0.0001	0.006	0.012	0.018
9.2	0.108	0.162	0.126	0.468	0.006	0.048	0.006	0.0162
11.0	0.444	0.876	0.138	1.266	0.288	0.276	0.036	1.320

The rate constants for each dye that are shown in **Table 4.3** indicate that myricetin photodegraded at the highest rate under all pH conditions while morin and kaempferol photodegraded at a significantly lower rate than myricetin, under all pH conditions. It is also worth noting that for all of the dyes in this study, the rate of loss of fluorescence increased significantly under more alkaline buffer conditions. The magnitude of the rate constants suggests that in a typical lab setting, a decrease in peak height using CE would likely be observed within 30 minutes or less.

The effect of time on the fluorescence signals of dyes that were separated using CE can be seen in **Figure 4.17**. In this figure, a sample containing 1.0 µg ml⁻¹ of myricetin, quercetin, kaempferol and morin was prepared in borate buffer (pH = 9.2) and kept in an open plastic centrifuge tube. The dye mixture was electrophoretically injected into a 50 cm (10 µm dia.) capillary and separated after 0, 15, 30, 105 and 180 minutes. In this experiment, a Solux bulb was not used, rather the dyes were simply exposed to the 50 W fluorescent lights used in the research laboratory.

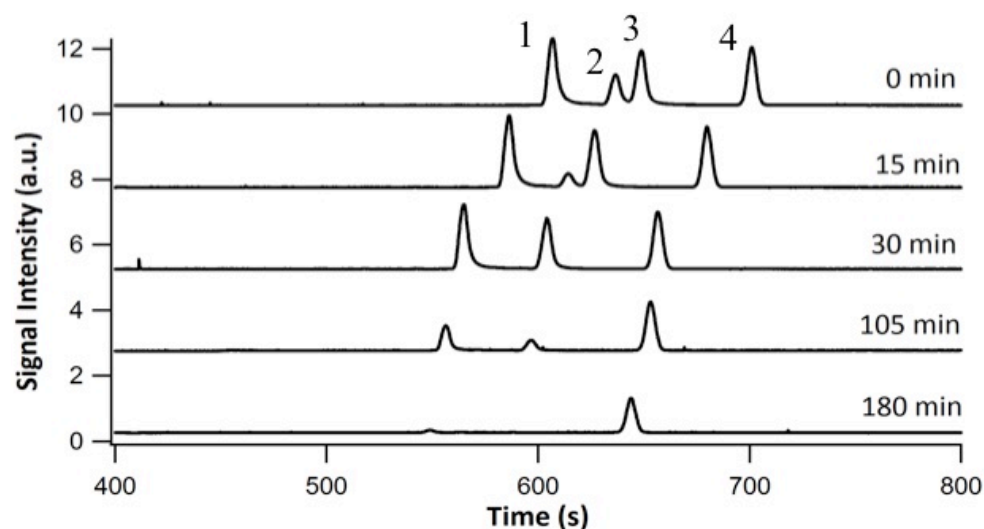


Figure 4.17 Separation of 4 flavonol dyes: (1) kaempferol, (2) myricetin; (3), quercetin; (4) morin. ($1 \mu\text{g ml}^{-1}$) at $\text{pH} = 9.2$. Each trace represents one separation after different time intervals. Separations were carried out using 20 mM borate buffer with 20 mM SDS and 20 kV applied voltage. Adapted from Ref. [22] – Copyright: Taylor and Francis

In **Figure 4.17** the rates of photochemical degradation of each dye can be observed by the decrease in their peak heights in the electropherograms over time. After 15 minutes, the size of the peak for myricetin decreased indicating that it is likely photodegrading at a higher rate than the other dyes. This is consistent with the kinetics experiments whereby myricetin had the highest rate constant. The electropherograms in **Figure 4.17** indicate that morin photodegrades at the slowest rate. This was not consistent with the kinetics experiment since kaempferol had a slightly smaller rate constant. After 30 minutes the peak for myricetin has almost completely disappeared and after 180 minutes, all of the peaks detected were either gone or significantly reduced.

According to the findings in Chapter 3, reasonably good separations were achieved at $\text{pH} = 8.5$. In order to determine if reproducible CE separations could be achieved over longer periods of time, a mixture of flavonols ($1 \mu\text{g ml}^{-1}$) was dissolved in 20 mM borate buffer at $\text{pH} = 8.5$. The same dye mixture was then separated repeatedly using CE after 0, 15 and 30 minutes and the

results of this experiment are shown in **Figure 4.18**. The results from this experiment indicate that these dyes are stable over a longer period of time with only a modest decrease in pH and the reproducibility of the peak heights for these separations is significantly improved. It should be noted, however that storing these dyes for longer periods of time (1-2 hours) will still likely result in their photodegradation, even at pH = 8.5.

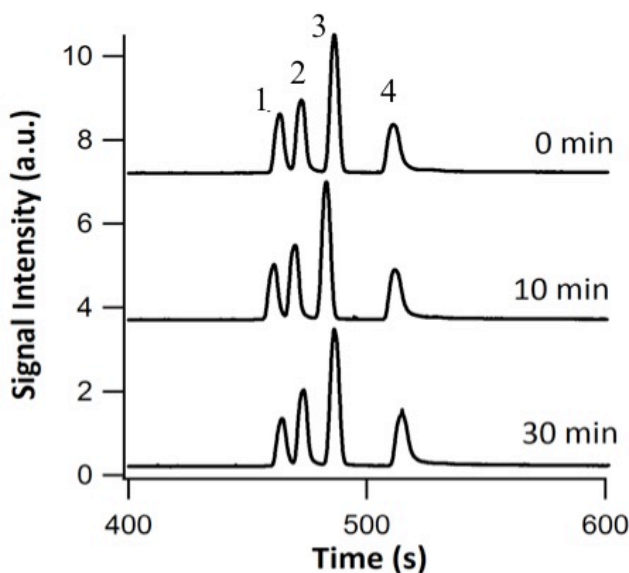


Figure 4.18 Separation of 4 flavonol dyes: (1) kaempferol, (2) myricetin, (3) quercetin and (4) morin ($1 \mu\text{g ml}^{-1}$) at pH = 8.5. Each trace represents one separation after a specified time interval. Separations were carried out using 20 mM borate buffer with 20 mM SDS and 20 kV applied voltage. Adapted from Ref. [22] – Copyright: Taylor and Francis

4.5 Conclusions

The objective of this study was to evaluate the relationship between the peak shape and peak reproducibility of purpurin and some flavonols with their photo-chemical behavior under the influence of pH in CE separation. The UV-Vis spectra of each class of dyes including: anthraquinones, flavones and flavonols were collected at pH values: 7.0, 9.2, and 11.0. These pH values were

chosen to have maximum and minimum criteria around the pH used in CE. The analysis of the changes in UV-Vis spectra of each class of dyes showed that under alkaline conditions the 60 minute old solution of purpurin the peak at 520 nm decreased drastically and the peak at 280 nm showed an increase in absorbance. It indicates that the chemical structure of purpurin has undergone the chemical changes. The increase in peak at 280 nm was in agreement with the absorbance of phthalic acid. The photodegraded products of purpurin were also identified by thin layer chromatography, NMR, and GC-MS, as: phthalic acid and salicylic acid.

Similar experiments were completed on flavonoids and it was concluded that at pH=11 the 60 minute old solution of myricetin did not show any absorbance at 450 nm as opposed other flavonols where at 450 nm their absorbance decreased. It was also observed that in all flavonols the peak at 324 nm appeared to increase. The peak could be assigned to hydroxyl benzoic acids, which were the products of decomposition of flavonols under alkaline conditions. The degraded compounds of flavonols were not identified in this work. It is recommended to identify degradation products in flavonols using the same techniques used for purpurin. The UV-Vis data showed that purpurin, quercetin and myricetin were the least stable dyes at alkaline conditions. To investigate the rate of decomposition of these dyes, the changes of their fluorescence intensity was measured over time. By plotting the natural log of fluorescence intensity of dyes as a function of time under different pH conditions it was concluded that all reactions followed the 1^o kinetics. The kinetics data also showed that the rate of decomposition of these dyes increased by exposing to light and higher pH value.

The results from this study would be used for defining the optimum pH for CE separation, while according to literature the best pH conditions for separating anthraquinones with CE is under

alkaline conditions. While these pH conditions (pH = 9.2) are well suited for most separations of anthraquinone dyes with CE, they are not for purpurin. Regarding flavonols the rate of their degradation (either oxidative or photochemical) is greatly increased under alkaline pH conditions. This experiment demonstrated that lowering the pH had a profound effect on separations with CE which resulted in the baseline separation of the mixture of anthraquinones as well as and flavonoids. The photostability of the flavonol dyes and purpurin as well as peak shape of purpurin could be improved dramatically by modest changes to pH of the running buffer.

Furthermore, the increased stability of purpurin at the lower pH (pH = 8.5) allowed for multiple separations to be carried out on the same solution with good reproducibility. Fluorescence detection is extremely sensitive, especially if a laser is used as a light source. The high sensitivity with laser induced fluorescence is critical in order to work with small samples. The best solution to minimize the photochemical decomposition of purpurin as well as flavonols is to run only fresh solutions, to store the standard in the dark and to carry out the CE separations at slightly lower pH with high applied voltages. Unfortunately, the downside of improving the stability of the dyes is the decreased ability to resolve peaks of flavonol dyes using CE.

4.6 References

1. Ahmadi, S., et al., *Photochemical properties of purpurin and its implications for capillary electrophoresis with laser induced fluorescence detection*. Dyes and Pigments, 2014. **105**: p. 57-62.
2. Trojanowicz, M., L. Wójcik, and K. Urbaniak-Walczak, *Identification of natural dyes in historical Coptic textiles by capillary electrophoresis with diode array detection*. Chemia analityczna, 2003. **48**(3): p. 607-620.

3. Maguregui, M., et al., *Micellar electrokinetic chromatography method for the determination of several natural red dyestuff and lake pigments used in art work*. Journal of Chromatography A, 2007. **1154**(1): p. 429-436.
4. López-Montes, A., et al., *Simultaneous identification of natural dyes in the collection of drawings and maps from The Royal Chancellery Archives in Granada (Spain) by CE*. Electrophoresis, 2007. **28**(8): p. 1243-1251.
5. Puchalska, M., et al., *Identification of anthraquinone coloring matters in natural red dyes by electrospray mass spectrometry coupled to capillary electrophoresis*. Journal of mass spectrometry, 2003. **38**(12): p. 1252-1258.
6. Sisa, M., et al., *Photochemistry of flavonoids*. Molecules, 2010. **15**(8): p. 5196-245.
7. Dall'Acqua, S., et al., *The photodegradation of quercetin: relation to oxidation*. Molecules, 2012. **17**(8): p. 8898-8907.
8. Craig, D., et al., *Fluorescence-based enzymatic assay by capillary electrophoresis laser-induced fluorescence detection for the determination of a few β -galactosidase molecules*. Analytical biochemistry, 1995. **226**(1): p. 147-153.
9. Derksen, G.C. and T.A. Van Beek, *Rubia tinctorum L*. Studies in natural products chemistry, 2002. **26**: p. 629-684.
10. Miliani, C., A. Romani, and G. Favaro, *Acidichromic effects in 1, 2-di-and 1, 2, 4-trihydroxyanthraquinones. A spectrophotometric and fluorimetric study*. Journal of Physical Organic Chemistry, 2000. **13**(3): p. 141-150.
11. Wang, D., G. Yang, and X. Song, *Determination of pKa values of anthraquinone compounds by capillary electrophoresis*. Electrophoresis, 2001. **22**(3): p. 464-469.
12. Al-Nuri, M.A., et al., *Emodin, a naturally occurring anthraquinone: its isolation and spectrophotometric determination in Rumex cyprius Plant*. Spectroscopy letters, 1996. **29**(8): p. 1539-1543.
13. Favaro, G., et al., *Role of protolytic interactions in photo-aging processes of carminic acid and carminic lake in solution and painted layers*. Journal of the Chemical Society, Perkin Transactions 2, 2002(1): p. 192-197.
14. Egerton, G. and A. Morgan, *The photochemistry of dyes II—some aspects of the fading process*. Coloration Technology, 1970. **86**(6): p. 242-249.
15. Hibbert, E., *Notes on the Effect of Light on Coloured Fabric—III*. Coloration Technology, 1929. **45**(7): p. 204-205.
16. Ahn, C. and S.K. Obendorf, *Dyes on archaeological textiles: Analyzing alizarin and its degradation products*. Textile research journal, 2004. **74**(11): p. 949-954.
17. Mabry, T.J., K.R. Markham, and M.B. Thomas, *The systematic identification of flavonoids*. 1970: Springer.
18. de Rijke, E., et al., *Analytical separation and detection methods for flavonoids*. J Chromatogr A, 2006. **1112**(1-2): p. 31-63.
19. Ramešová, Š., R. Sokolová, and I. Degano, *The study of the oxidation of the natural flavonol fisetin confirmed quercetin oxidation mechanism*. Electrochimica Acta, 2015. **182**: p. 544-549.

20. Monici, M., et al., *Flavone photoreactivity. UV-induced reactions in organic solvents and micellar systems*. Journal of Photochemistry and Photobiology B: Biology, 1993. **20**(2-3): p. 167-172.
21. Jurasekova, Z., et al., *Effect of pH on the chemical modification of quercetin and structurally related flavonoids characterized by optical (UV-visible and Raman) spectroscopy*. Physical Chemistry Chemical Physics, 2014. **16**(25): p. 12802-12811.
22. Goltz, D., et al., *Photochemical properties of selected flavonol dyes: Effects on their separation using capillary electrophoresis*. Journal of Liquid Chromatography & Related Technologies, 2016. **39**(16): p. 768-774.

Chapter 5

Identification of dyes in genuine textile
samples by CE-LIF

5.1 Introduction

In most textiles, dyes are bonded to the substrate with a mordant, a metal which has the role of a ‘connector’ between the dye and the textile. Dyes adhere to the substrate by intermolecular forces of attraction such as Van der Waals and dipole-dipole interactions. In order to extract dyes from substrates including textiles and paintings, a proper solvent should be used. If the textile is mordanted the dye can be extracted using acid to hydrolyse the bond between the dye and metal, which is considered to be a ‘harsh’ method. Conversely, a ‘mild’ extraction refers to the application of organic solvents [1]. Experimental conditions of extraction such as: temperature, time, and agitation (vortexing or using ultrasonication) also can affect the extraction efficiency. The optimum temperature and extraction time were defined from the results obtained from analytical methods performed on the textile samples to achieve extracts.

In these extraction methods time and temperature were manipulated and the amount of extract from each experimental condition was evaluated. A favorable temperature should be below the boiling point of the solvent, to avoid vaporization of the solvent in an open tube. This also prevents the loss of the microsamples as well as the extract through pressure build up in a closed tube. Also, the optimum extraction time can be evaluated by the analytical method, when the most extract is achieved. A literature review was done on the extraction methods used by other research groups for dyes and different textiles. The most common solvents used for extraction were H_2SO_4 , HCl (strong acid), HCOOH (weak acid), methanol, pyridine, dimethyl sulfoxide, and dimethylformamide.

The dyes in a big chunk of red and yellow samples belonging to 16-17th century were extracted using few drops of strong H₂SO₄ and were studied by TLC [2]. Hydrochloric acid has been used in different concentrations and conditions: For example, HCl 6 N at 20°C for 6 hours was used to extract dyes from red samples belonging to the 16th century and the presence of lac dye; pseudo purpurin, alizarin and munjistin; carminic acid were detected using TLC [3]. Red dyes were also extracted from 2 mg samples using HCl 3N at 100°C for 30 minutes and running HPLC-DAD could confirm the presence of alizarin, pseudo purpurin, purpurin, rutin, kermesic acid, alizarin and purpurin [4]. The application of the mixture of HCl with methanol, and HCl with ethanol at different v/v ratios were reported. The range of temperatures and time for extractions were 60-100°C, 10-30 minutes [5-19]. The results from these studies showed that the application of methanolic HCl was preferable to extract red dyes since they are mostly aglycone and did not hydrolyse with acid as opposed to yellow dyes. It was also reported that HCl based extraction can destroy the fiber samples, based on the concentration, as well as the experimental conditions [19]. Therefore, some mild extractions were developed including the application of formic acid, and other organic solvents. A mixture of formic acid and methanol was used by others and the temperature and the time of extraction were in the following ranges: 40-100°C and 5-30 minute [12], [15], [18], [19]. Based on the findings from these studies, formic acid was more favorable for extracting of glycone flavonoids as the glycosides were intact. Moreover, pyridine, dimethyl sulfoxide, and dimethylformamide were used for dyes' extraction. The application of a mixture of pyridine and water at 90°C for 1 hour on 10 mg red and yellow samples was reported [20]. Dimethyl sulfoxide was used at 100°C on 1-2 mg samples dyed with weld and all glycone and aglycone flavonoids

were detected using HPLC-DAD [15]. A few articles on the study of dyes extracted from textiles and paintings using CE equipped with other detection systems have been published. The extraction method used in these studies was different: e.g. for the study of dyes from a map extraction was achieved using a brush imbedded with 0.1 M SDS, directly applied on the map surface. The red dye was identified as carminic acid. Two yellow samples were extracted from different locations on the map. A very small peak of gambogic acid could be detected in one of the yellow samples. In another one crocetin and gambogic acid were detected where the peak assigned for crocetin was a poor- shaped peak [21]. The application of H_2SO_4 1 M has been reported for the acid hydrolysis of dyes from the small red samples from a 17th century oil painting. The sample and acid were kept under mechanical shaking for 15 minutes; the residue was extracted with dichloromethane applying mechanical shaking. The solution was left under N_2 stream at 60°C to dryness following dissolving into the electrolyte for CE-DAD study. Three small poorly shaped peaks in the electropherogram of the red sample were assigned as alizarin, quinizarin and purpurin [22].

A summary of this survey is presented in Appendix B **Table B5.1**, indicating different solvents used for extraction of dyes as well as the conditions of application including: concentration of solvent, temperature and time. The qualities of each sample (wool/silk, colour) and the results after analysis are given. **Table B5.1**. This table assisted in choosing a selection of solvents used for extraction of red and yellow dyes. The list of the compounds and their abbreviations introduced in list of symbols (page XXIV).

From this survey, it was concluded that despite the important role of extraction in study of the micro samples, it has not been investigated systematically. Since each dye has unique chemical structure and properties, there is no one single extraction method. Therefore, some research groups

have investigated different solvents for the extraction of dyes. Time and temperature were also manipulated to achieve the optimum conditions for extraction. However, other research groups based on the similarity between their work, mimic the same extraction method introduced by others without maneuvering the parameters. In this project, for the first time CE-LIF was used to identify dyes extracted from textiles. Therefore, in order to find out the most effective extraction procedure compatible with CE-LIF, the extraction methodology was designed based on the several common extraction procedures used in different analytical approaches.

The goal of the research presented in this chapter is to examine the efficiency of different solvents for the extraction of madder and weld from the laboratory dyed wool samples using the solvents selected from the literature. The results on the original samples belonged to different eras: 7th BC, 17th and 21th AD. The extracted dyes were analyzed using CE-LIF and separation conditions were defined based on the results obtained from Chapter 3: using 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

The experimental design for this chapter is as follow: the wool honks were dyed using madder and weld, the procedure is given in next section: “preparation laboratory samples dyes with madder and weld”. The dyes were extracted from 1mg wool samples using a list of solvents selected from literature by manipulating extraction parameters: time, temperature and ultrasonication. The number of solvents were narrowed down based on the observation of changes in colour of extract solution and the wool samples. The results from observation were verified using CE-LIF. The results presented here is the representative of running CE-LIF on 3 replicates of the extract solution.

It should be noted that the migration time of the dyes extracted from the samples were different with those of standard solutions. It can be due to the extraction of unwanted compounds from the samples where could not be separated from the dyes by filtration.

5.2 Evaluation of 15 solvents

Based on the information obtained from the literature review, extraction experiments were designed to evaluate the effect of different solvents as well as extraction conditions on the red and yellow laboratory prepared textiles dyed with madder and weld.

For this research 8 solvents were chosen, and H_2SO_4 , HCl and HCOOH were used at different concentrations as a mixture with other solvents such as methanol (MeOH), ethanol (EtOH), distilled water (W), and acetonitrile (ACN). The solvents used for this research are as follows:

1. HCl 37%, HCl 37%: MeOH : W (2:1:1, v/v), HCl 3N, HCl 3N: (EtOH) (1:1, v/v)
2. H_2SO_4 10%, H_2SO_4 10%: EtOH (1:3, v/v)
3. HCOOH : MeOH (5:95, v/v), HCOOH : MeOH : W (2:1:1, v/v/v)
4. MeOH : W (1:1, v/v)
5. EDTA 0.001 M: ACN : MeOH (1:5:44, v/v/v)
6. Pyridine
7. DMSO
8. DMF

The extraction was performed on samples at 60°C and 90°C , for 5 and 10 minutes for harsh extractions, and 15 and 30 minutes for mild extractions. All experiments were performed in 2

replicates to investigate the effect of ultrasonication. By considering the number of parameters to investigate, 104 red and 104 yellow dyed samples were needed for each batch of experiments.

5.3 Preparation of laboratory samples dyed with madder and weld

Coarse wool hanks, madder, weld, all mordants salts and Potassium bitartrate, known as potassium hydrogen tartrate or cream of tartar, were purchased from Grant Bazar in Tehran and the wool was dyed according to Scheweppe's recipe [23]. The wool was washed, mordanted and dyed in 3 steps.

Washing step

Ten 100.0 g of wool hanks were washed thoroughly with detergent to remove spinning oil. They were soaked in 30-40°C bath for 2 hours then they were drained and immersed into another 30-35°C bath containing detergent. The hanks were washed, drained and left to air dry.

Mordanting process

The wool hanks were mordanted prior to dyeing. The salts used as mordants for madder dyeing were: hydrated potassium aluminium sulfate ($\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$) known as alum, tin II chloride ($\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$) known as 'tin', iron II sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) known as 'iron', copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) known as 'copper' and potassium dichromate; $\text{K}_2\text{Cr}_2\text{O}_7$ known as 'chrome'. Alum was the only mordant used for dyeing with weld. For each mordant salt a 40°C water bath containing 2.5-3.5 L was used. Specific amounts of each salt were dissolved in 100 ml of hot water and

added to the water bath except for Fe^{+2} which was added directly to the dye bath. The wet hank wool was immersed into the mordant bath and the temperature of the bath was increased from 40°C to 90°C in one hour. The wool stayed in the mordant solution for specific time: In case of alum, chrome, and tin the immersing time was 60 minutes. While for iron and copper the immersing time was 30 minutes and 90 minutes, respectively. After that the wool samples were taken out and left to air dry.

The amount of mordant salts used for 100.0 g of hank wools were as follows: alum: 15.0 g alum with 6.0 g of cream of tartar (potassium hydrogen tartrate, $\text{KC}_4\text{H}_5\text{O}_6$), chrome: 3.0 g of chrome, iron: 3.0 g iron with 6.0 g of cream of tartar, copper: 6.0 g, tin: 3.0 g tin, 3.0 g cream of tartar and 3.0 g oxalic acid.

Dyeing process

Weld (300 g) was pulverised and wrapped in the cotton cloth and it was suspended in 3 L water bath for about 6 hours. The temperature was increased until the water boiled and the weld wrap was left at 80°C for 45 minutes. Then it was taken out, and the temperature of dye bath dropped to 35-40°C, next wet alum mordanted wool was immersed into the dye bath and the temperature was increased to 80°C and the wool was left there for 60 minutes.

In the case of madder, 100 g of pulverised madder was wrapped in a cotton cloth and it was soaked in 40°C, 6 L water bath for 12 hours. Next, in one hour the temperature of the dye bath was increased to boiling point and the madder wrap was left 10 minutes at this temperature. Then it was taken out, and the temperature of dye bath dropped to 40°C. Later, wet mordanted wool was

immersed into the dye bath and the temperature was increased to 80°C and the wool was left there for 30 minutes.

After the temperature of the dye bath was dropped to 35-40°C the wool was removed and it was rinsed and left to dry at room temperature.

5.4 Visual evaluation of the extraction efficiency

The extraction efficiency of 15 solvents on madder and weld dyed samples was evaluated visually. Red and yellow samples were cut in 1.000 ± 0.001 mg. They were transferred to the 1.5 ml test tubes and 60 μ l of the solvent was added to each sample and hydrolysed under specific conditions. The test tubes were labeled as 1, 2, 3, 4, 1U, 2U, 3U, 4U where: 1, defines a 1.000 ± 0.001 mg of a sample hydrolysed in a 500 μ l solvent, at 60°C, for 5 minutes. Code 1U shows the same extraction procedure as 1, including using ultrasonication. Using underline in coding refers to longer extraction time. **Table 5.1** shows the summary of the labels used for the samples.

Table 5.1 Introducing the labels used for the extraction condition applied on the samples

label	Temperature (°C)	time (minute)		ultrasonic wave (U)
		harsh extraction	mild extraction	
1	60	5	15	n/a
<u>2</u>	60	10	30	n/a
3	92	5	15	n/a
<u>4</u>	92	10	30	n/a
1U	60	5	15	U
<u>2U</u>	60	10	30	U
3U	92	5	15	U
<u>4U</u>	92	10	30	U

In order to evaluate the extraction method, 2 parameters were checked: comparing the colour of the wool sample before and after extraction, and checking the colour of extraction solutions or the dye residue after drying the solution. Therefore, the methods that discoloured wool samples more and gave stronger solutions were chosen for CE study. Based on the following observation, the extraction methods were chosen for CE.

Wool samples dyed with madder

HCl 37%: All substrates were destroyed by this solution and the residues inside the test tube (were indivisible mixtures of digested wool sample and red dye).

HCl: MeOH: W (2: 1: 1, v/v/v): The wool samples at 60°C were grey and at 92°C were black and brittle. The colour of extraction solutions at 60°C was bright red and by increasing the heat it got deeper. The best result was achieved at 60°C for 10 minutes (2).

HCl 3N: The samples remained intact and their colour changed to very light orange. Using ultrasonication did not improve the extraction and destroyed the samples. It seems using 92°C, with the extraction time of 5 minutes gave the best result (3).

HCl 3N: EtOH (1:1, v/v): The presence of ethanol improved the extraction results. These samples were much lighter than the samples from HCl 3N. The best extraction was obtained using 92°C, with the extraction time of 5 minutes (3).

H₂SO₄ 10%: Sulfuric acid digested all samples and the solution in all test tubes was clear. No methods were chosen.

H₂SO₄ 10%: EtOH (1:3, v/v): Similar to H₂SO₄ 10%, all samples were destroyed, but adding ethanol to sulfuric acid increased the extraction efficiency which can be due to ‘leveling effect’ of ethanol on sulfuric acid [24]. Leveling effect refers to the impact of the solvents on the strong acid

or strong base. While base is added to a strong acid solution the strength of acid is leveled or by adding acid to the strong base solution, the basicity of the solution is leveled. The solutions were tinged yellow-orange in comparison to the clear solutions obtained using H₂SO₄ 10%. It was concluded that by increasing heat and time of extraction, the results were improved. No conditions were chosen.

HCOOH: MeOH (5:95, v/v): The extraction solution was dried in less than 12 hours. All samples were faded to the same extent at 60 and 92°C. Using ultrasonication improved the results. The best result was achieved using 92°C, 15 minutes extraction time using ultrasonication (2U).

HCOOH: MeOH: W (2:1:1, v/v/v): It was concluded that by increasing the pH, the extraction did not improve. All samples were faded and the solutions were orange. Based on the colour of the samples and the solutions, method 3U was chosen: 92°C, 15 minutes extraction time including ultrasonication.

MeOH: W (1:1, v/v): As opposed to the samples extracted with HCl and H₂SO₄, all samples were intact. It was concluded increasing the temperature and time increased the discolouration of the wool samples. The colour of the extraction solutions were kind of clear. The recommended condition was extraction at 92°C for 30 minutes, including ultrasonication (4U).

EDTA 0.001 M: ACN: MeOH (1:5:44, v/v/v): The colour of the wool samples did not change and it was the same as before extraction. The extraction solution evaporated during the night and there was no colour residue left behind. No extraction condition was chosen.

Pyridine: Small changes in colour of the samples could be observed. The solutions were clear. Using higher temperature and ultrasonication improved the results. The solution from method 4U

showed a little stronger red solution to compare to the rest. The recommended condition was extraction at 92°C for 30 minutes, including ultrasonication (4U)

DMSO: The best result was achieved using 92°C for 30 minutes, including ultrasonication (4U).

DMF: The best result was achieved using 92°C for 30 minutes, including ultrasonication (4U).

Wool samples dyed with weld

HCl 37%: The strong acid had destroyed all samples and it was not possible to separate the digested samples from yellow bright solutions.

HCl: MeOH: W (2:1:1, v/v/v): The extraction solutions were less yellow to compare the solutions obtained using HCl 37%. The wool samples after extraction at 92°C were brittle, similar to the results observed in samples dyed with madder. The samples treated with ultrasonication could not be separated from the solution. Using a centrifuge followed by using filter tubes also did not improve the separation phases. Method 2 (60°C, 10 minutes) was chosen.

HCl 3N: All samples did not show any changes in their colour and the solutions were clear. Method 2/3U was chosen. (60°C, extraction time of 10 minutes, including ultrasonication)

HCl 3N: EtOH (1:1, v/v): The results of extraction were improved using higher temperature and applying ultrasonication. All solutions were clear the same as solutions obtained using HCl 3N. The extraction method of 4U was chosen. (92°C, extraction time of 10 minutes, including ultrasonication)

H₂SO₄ 10%: All wool samples were digested and very weak yellow solutions were obtained, which were inseparable from each other.

H₂SO₄ 10%: EtOH: All samples were digested, and the bright yellow solutions were achieved. The intensity of the yellow colour was similar to the solutions achieved using HCl: MeOH: W. The digested samples could not be separated from their solutions.

HCOOH: MeOH (5:95, v/v): The wool samples were intact however faded by treatment but the solutions were still clear. The extraction efficiency was increased using higher temperature, longer extraction time and applying agitation. But, using higher temperature and longer extraction time ended up bursting the test tubes and the samples and extraction solutions were lost. Therefore, method 2U was chosen: 60°C, 30 minutes, using ultrasonication.

HCOOH: MeOH: W (2:1:1, v/v/v): The samples faded but they still showed a tint of yellow colour. The solutions were less yellow than the solutions obtained using HCOOH: MeOH (5:95). It was observed that increasing the extraction time did not improve the results, as opposed to using ultrasonication. Since the samples were burst at 92°C, method 3/2U was chosen (92°C, 15 minutes, and using ultrasonication).

MeOH: W (1:1, v/v): All wool samples were intact. Increasing the temperature did not improve the extraction results. All solutions were clear except for 4U: extraction at 92°C, 30 minutes, using ultrasonication.

EDTA 0.001 M: ACN: MeOH (1:5:44, v/v/v): Similar to extraction of madder samples, there was no observation of colour changing in samples or solutions after extraction.

Pyridine: The samples were intact after extraction and the solutions were weak yellow. Using ultrasonication improved the extraction. Method 4U was chosen: extraction at 92°C, 30 minutes, using ultrasonication.

DMSO: The samples were yellow and the solution was weak yellow. The best results were achieved using 4U: extraction at 92°C, 15 minutes, using ultrasonication.

DMF: The samples were yellow and the solution was weak yellow. The best results were achieved using 4U: extraction at 92°C, 30 minutes, using ultrasonication.

In following the details of the observations of each extraction method are documented. **Table 5.2 and 5.3** presents the summary of the whole set of extractions performed on red and yellow samples. The cells highlighted refer to the methods that were chosen for CE studies.

Table 5.2 The summary of extraction methods applied on the yellow wool samples dyed with madder

	Temperature (°C)	60	<u>60</u>	92	<u>92</u>	60	<u>92</u>	92	<u>92</u>
	Time (minutes)	5	<u>10</u>	5	<u>10</u>	5	<u>10</u>	5	<u>10</u>
	Ultrasonication (U)	—	—	—	—	U	<u>U</u>	U	<u>U</u>
Solvent									
1	HCl 37%	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
2	HCl: MeOH: W (1:1:1, v/v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
3	HCl 3N	1	<u>2</u>	<u>3</u>	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
4	HCl 3N: EtOH (1:1, v/v)	1	<u>2</u>	<u>3</u>	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
5	H ₂ SO ₄ 10%	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
6	H ₂ SO ₄ 10%: EtOH (1:3, v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
	Temperature (°C)	60	<u>60</u>	92	<u>92</u>	60	<u>60</u>	92	<u>92</u>
	Time (minutes)	15	<u>30</u>	15	<u>30</u>	15	<u>30</u>	15	<u>30</u>
	Ultrasonication (U)	—	—	—	—	U	<u>U</u>	U	<u>U</u>
Solvent									
7	HCOOH: MeOH (1:1, v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	<u>3U</u>	<u>4U</u>
8	HCOOH: MeOH: W (2:1:1, v/v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	<u>3U</u>	<u>4U</u>
9	MeOH: W (1:1, v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3 U	<u>4U</u>
10	EDTA 0.001 M: ACN: MeOH (1:5:44, v/v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
11	Pyridine	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
12	DMSO	1	<u>2</u>	3	<u>4</u>	1 U	<u>2U</u>	3U	<u>4U</u>
13	DMF	1	<u>2</u>	3	<u>4</u>	1 U	<u>2U</u>	3U	<u>4U</u>

Table 5.3 The summary of extraction methods applied on the red wool samples dyed with weld

	Temperature (°C)	60	<u>60</u>	92	<u>92</u>	60	<u>60</u>	92	<u>92</u>
	Time (minutes)	5	<u>10</u>	5	<u>10</u>	5	<u>10</u>	5	<u>10</u>
	Ultrasonication (U)	—	—	—	—	U	<u>U</u>	U	<u>U</u>
Solvent									
1	HCl 37%	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
2	HCl: MeOH: W (1:1:1, v/v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
3	HCl 3N	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
4	HCl 3N: EtOH (1:1, v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
5	H ₂ SO ₄ 10%	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
6	H ₂ SO ₄ 10%: EtOH (1:3, v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
	Temperature (°C)	60	<u>60</u>	92	<u>92</u>	60	<u>60</u>	92	<u>92</u>
	Time (minutes)	15	<u>30</u>	15	<u>30</u>	15	<u>30</u>	15	<u>30</u>
	Ultrasonication (U)	—	—	—	—	U	<u>U</u>	U	<u>U</u>
Solvent									
7	HCOOH: MeOH (1:1, v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
8	HCOOH: MeOH: W (2:1:1, v/v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	<u>3U</u>	<u>4U</u>
9	MeOH: W (1:1, v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3 U	<u>4U</u>
10	EDTA 0.001 M: ACN: MeOH (1:5:44, v/v/v)	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
11	Pyridine	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
12	DMSO	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>
13	DMF	1	<u>2</u>	3	<u>4</u>	1U	<u>2U</u>	3U	<u>4U</u>

5.5 CE-LIF and extraction efficiency

As mentioned previously, the dye was extracted in the sample using different solvents and CE-LIF was performed to study the extraction efficiency of the selected 6 representative solvents.

5.5.1 Sample preparation for CE injection

The residue of dye extract in the test tube was dissolved by adding 5 μl of DMSO and vortexing for 1 minute. The test tube was centrifuged to sediment the undissolved residue. In order to prepare the sample for CE injection between 1 to 5 μl of solution (based on the colour intensity of the solution) was added to the borate buffer pH = 8.5 which in total had 5% acetonitrile and 10% (v/v) DMSO in total volume of 25 μl . It should be mentioned since sensitivity of the CE fluctuated daily, the amount of the spike was checked based on the fluorescence intensity of 5 $\mu\text{g ml}^{-1}$ standard solution of dye daily. As mentioned before, the dye was extracted in the sample using different solvents and the efficiency of the extraction method was studied using CE-LIF. The amount of the spike was checked based on the fluorescence intensity of 5 $\mu\text{g ml}^{-1}$ standard solution of dye daily.

5.5.2 The effect of DMSO on migration time

It is worth mentioning that the standard solution of dye was in DMSO and spiking changed the DMSO percentage, which affected the dyes migration time after spiking. **Figure 5.1** shows the relationship between current and DMSO concentration in a 20 mM borate buffer containing 20 mM SDS and 5% acetonitrile using 22 kV applied voltage. It can be concluded that by each 1%

DMSO increment, the current dropped by 5%, which resulted in an increase in the migration time of the eluents.

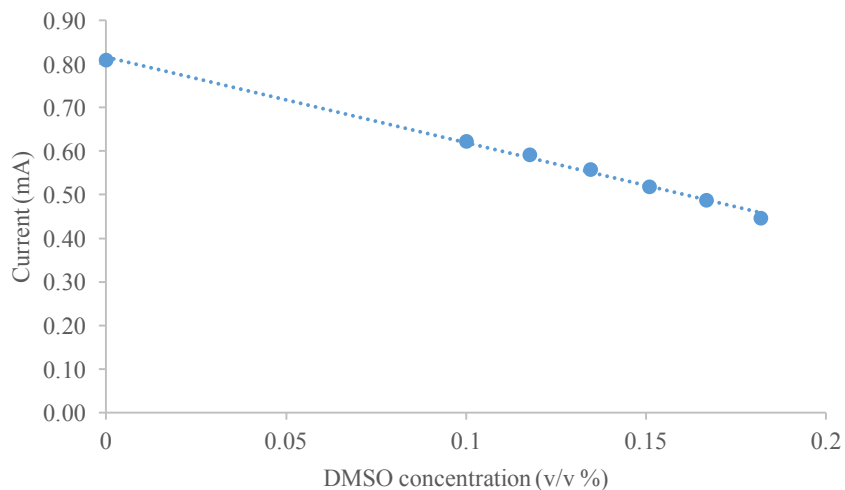


Figure 5.1 The changes of current (mA) as a function of DMSO concentration (v/v %) in 20 mM borate buffer at pH = 8.5 with 20mM SDS and 5% acetonitrile under 22kV

5.5.3 Hansen solubility parameter

In this work, extraction was affected by 4 parameters: solvent, temperature, time and mechanical agitation. **Table B5.1** represents the application of popular solvents for extraction dyes from textiles. In the literature, the reasons for choosing the solvents for extraction of dyes from textiles has not been explained. Moreover, how different solvents can result in different extraction efficiency has also not been considered in detail.

Regarding the effect of the solvents on extraction, Hansen solubility parameter can be used to explain the reason why some solvents could extract more dyes than the others.

The Hansen solubility index is based on 3 molecular interaction forces: van der waals interaction force, static electronic interaction force, and hydrogen bonding, which are equivalent to dispersion

energy (D), polar energy (P) and hydrogen bonding energy (H) respectively. The amount of energy required to overcome these molecular forces to evaporate the solvent is known as cohesive energy (E_{coh}) [25]. Therefore, cohesive energy is defined as Eq. 5.1.

$$E_{coh} = D + P + H \quad \text{Eq. 5.1}$$

On the other hand, cohesive energy is equal to the heat of vaporization, which is defined as in Eq. 5.2:

$$E_{coh} = H_V - RT \quad \text{Eq. 5.2}$$

Where E_{coh} = cohesive energy, $J \text{ mol}^{-1}$, H_V = the heat of evaporation, $J \text{ mol}^{-1}$

R = the universal gas constant, $8.314 J \text{ mol}^{-1} K^{-1}$, T = the absolute temperature, K

Since the cohesive energy is not proportional to the size of the molecules (it means, the bigger molecules do not have the higher cohesive energy), it is used as cohesive energy density, which is the ratio of cohesive energy to molar volume (Eq. 5.3):

$$\frac{E_{coh}}{V} = \frac{H_V - RT}{V} = \frac{D}{V} + \frac{P}{V} + \frac{H}{V} \quad \text{Eq. 5.3}$$

E_{coh} = cohesive energy, $J \text{ mol}^{-1}$, V = molar volume, $cm^3 \text{ mol}^{-1}$

Hilderbrand introduced the solubility parameters δ_{tot} as the square root of cohesive energy density (Eq. 5.4)

$$\delta_{tot} = \sqrt{\frac{E_{coh}}{V}} \quad \text{Eq. 5.4}$$

δ_{tot} = Hilderband solubility parameter, $MP^{1/2}$

Therefore, the solubility parameter can be introduced as (Eq. 5.5)

$$\delta_{tot} = \sqrt{\delta D + \delta P + \delta H} \quad Eq. 5.5$$

These 3 parameters can be used as 3 coordinates to depict a point in space. Plotting the 3 pairwise showed that δP vs δH was spherical as opposed to δH vs. δD and δP vs. δD . Hansen modified this by adding the factor of 4 in δD as Eq. 5.6:

$$\delta_{tot} = \sqrt{4 \delta D + \delta P + \delta H} \quad Eq. 5.6$$

These parameters 4 $\delta D, \delta P, \delta H$ can be used as coordinates for a point in space, which gives the Hansen sphere. If 2 molecules have similar HSP they are alike and are soluble with each other or they are miscible. In order to find out if 2 molecules are compatible, R_o parameter, interaction radius of the solute is used. It is the radius of the sphere belonging to the solute in Hansen space and is defined through the results from testing the solubility of the solute in a large number of solvents [26]. Therefore, a solvent can dissolve a solute if its Hansen solubility parameters lie within the solute's parameters. The distance between HSP values of solvent and solute known as R_a solubility parameter distance, defined as Eq. 5.7:

$$R_a = \sqrt{4(\delta D_1 - \delta D_2)^2 + (\delta P_1 - \delta P_2)^2 + (\delta H_1 - \delta H_2)^2} \quad Eq. 5.7$$

Where: R_a = distance between solvent and solute solubility sphere, $MP^{1/2}$

δX_1 = solute Hansen component parameter

δX_2 = solvent Hansen component parameter

if R_a is small, it means that the solvent and solute are alike and solvent can dissolve the solute, the smaller R_a , the better solvent.

The ratio of R_a to R_o known as relative energy difference (RED) is shown as (Eq. 5.8)

$$RED = \frac{R_a}{R_o} \quad Eq. 5.8$$

Where: RED = Relative energy difference, R_a = solubility parameter distance, $MP^{1/2}$

R_o = radius of a Hansen solubility sphere, $MP^{1/2}$

If $RED < 1$ it means the solvent is more effective in dissolving the solute, as opposed to $RED > 1$ which means the solvent has no effect [27].

In this work, for the first time Hansen solubility parameters are used to compare the effect of different solvents in extraction of dyes from wool samples. In order to evaluate the most effective solvent for dissolving alizarin, the R_a distance between alizarin and the solvents were calculated using Eq.5-5. The estimation of HSPs of alizarin was taken from literature [28], and the HSP values of the solvents were obtained from *Hansen Solubility Parameters A User's Handbook, 2nd edition*. [26]. The HSPs of luteolin and apigenin were kindly provided by Dr. Steven Abbott by using HSPiP's automatic system (personal communication, March 4, 2015). These HSPs are given in **Table 5.4**. Also, the distances (R_a) of alizarin with all solvents are compared in Table 5.4. The R_a values are used to compare the solubility of alizarin, luteolin and apigenin in different extraction solvents. The R_a values are used to compare the solubility of alizarin, luteolin and apigenin in different extraction solvents.

Table 5.4 The HSP values of alizarin, luteolin, apigenin and the solvents, as well as the R_a distance between these dyes and the solvents

Compound	δ_D	δ_P	δ_H	R_a	R_a	R_a
				alizarin	luteolin	apigenin
alizarin	21.0	11.3	22.0			
luteolin	20.8	5.9	9.5			
apigenin	20.5	5.8	12.1			
formic acid	14.3	11.9	16.6	14.4	16.0	14.5
water	15.6	16.0	42.3	23.4	35.9	33.3
formic acid: MeOH (5:95, v/v)	15.1	12.3	22.0	11.8	18.1	16.0
formic acid: MeOH: W (2:1:1, v/v)	14.8	13.0	24.5	12.6	20.4	18.3
methanol	15.1	12.3	22.3	11.8	18.3	16.2
methanol: W (1:1, v/v)	15.4	14.2	32.3	15.5	26.6	24.2
pyridine	19.0	8.8	5.9	16.8	5.9	7.5
dimethylformamide (DMF)	17.4	13.7	11.3	13.1	10.5	10.1
dimethylsulphoxide (DMSO)	18.4	16.4	10.2	13.8	11.6	11.6

5.5.4 Extraction efficiency of madder dyed samples using CE-LIF

CE-LIF was performed to evaluate the extraction efficiency of alizarin using selected 6 representative solvents including: HCl, formic acid, methanol, pyridine, DMSO and DMF.

Hydrochloric acid (HCl) extraction of wool samples dyed with madder

From the literature review it was concluded that HCl, at different concentrations and different mixtures with alcohol, is the most common solvent for extraction of red dyes. The application of HCl at 2, 3, and 6 M under different conditions to extract red dyes from textiles has been reported by different researchers. Karadağ *et al.* used HCl 6M at 20°C for 6 hours to extract the red dyes from 16th century red textiles. Lac dye, pseudo purpurin and carminic acid were identified by using TLC and UV spectroscopy in 3 samples respectively [3]. Wouters used 3M HCl at 100°C for 30 minutes and by using HPLC-DAD alizarin; pseudo purpurin; munjistin; purpurin and rubidian, kermesic acid and alizarin, carminic acid and kermesic acid were identified in 3 red textiles respectively [4]. Kharbade *et al.* used HCl 2M to extract the different dye components from Indian textiles belong to the 16-19th century using TLC. Only red dyes such as alizarin and pseudo purpurin could be identified [29]. Regarding HCl with the mixture of alcohols, it can be noted that the most widespread mixture is HCl 37%: MeOH: W (2,1,1, v/v/v). This mixture has been used for decades to extract mostly red dyes and aglycone precursors of yellow dyes from textiles. Clementi *et al.* stated that HCl extraction is the most effective method to extract colouring matters from madder [30]. The extraction can be affected by time and applied temperature, in most studies the applied extraction conditions were: 100°C for 10 minutes [5], [10], [11], [12], [13], [19], or 105°C for 10 minutes [16], [17] and 60°C for 30 minutes [18]. A lower concentration of HCl with methanol (HCl 3M: MeOH, 1:1, v/v) at 100°C for 15 minutes has been used [6]. Moreover, Surowiec *et al.* and Trojanowicz *et al.* extracted dyes from Coptic textiles belonging to the 4-5th and 7-9th centuries by a mixture of HCl 3M with ethanol (1:1, v/v) at 90°C for 20 minutes [8], [9]. Surowiec

et al. used HPLC-FLD and Trojanowicz *et al.* performed CE- DAD on the same extract. Application of both techniques could be used to identify the presence of purpurin, kaempferol in a black sample, while munjistin could be identified using HPLC-FLD and alizarin using CE-DAD.

In this work, the dyes were extracted using HCl 3M, HCl 3M: EtOH (1:1, v/v) at 92°C for 5 minutes and HCl 37%: MeOH: W (2:1,1, v/v/v) at 60°C for 10 minutes. The extracted dyes were studied using CE (**Figure 5.2**). In these 3 separations, by spiking with alizarin standard solution in DMSO (run a-1 in embedded Figure) peak 1 was identified as alizarin. The comparison between the runs shows that 3N HCl in the presence of ethanol (run c) was more effective to extract alizarin than other 3N HCl applications: by itself (run b), or the mixture of HCl with methanol and distilled water (run a).

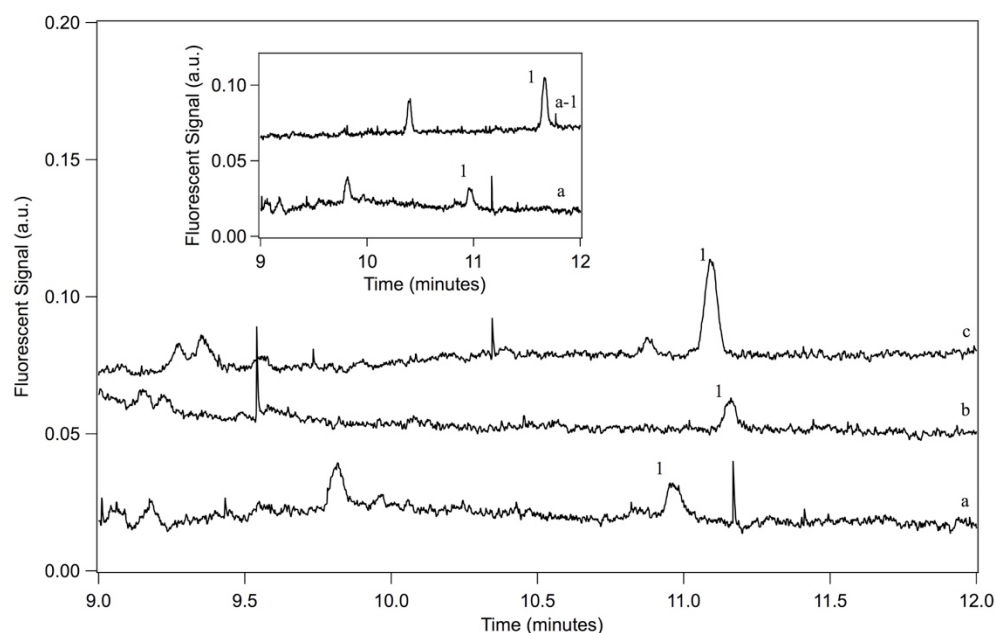


Figure 5.2 Electropherograms of dye extract from wool samples dyed with madder, using HCl: (a) HCl: methanol: distilled water (1:1:1, v/v/v), (b) HCl 3N, (c) HCl 3N: ethanol (1:1, v/v). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

These results are in accordance with our observation of choosing the solvent for extraction and in agreement with Orska-Gawry *et al.* that replacing methanol with ethanol due to higher B.P increases the extraction time and in return increases the amount of extracted dyes [7]. It should be mentioned that alizarin in the standard solution with the same ionic strength as in the extract dye solution (containing 5% acetonitrile, 10% DMSO), eluted around 8 minute (results from Chapter 1). However, the delay observed in HCl extractions can be due to the presence of the unwanted residue extract from wool samples. Surowiec *et al.* could extract dyes using the mixture of HCl 3M: EtOH (1:1, v/v) by performing CE- DAD alizarin and purpurin could be detected [8].

Formic acid (HCOOH) extraction of wool samples dyed with madder

Formic acid is another common solvent used for extraction of dyes from textiles typically used as a mixture with methanol at (5:95, v/v). According to the findings of the literature review extraction conditions were applied at different temperatures for different times; for example, 40°C for 30 minutes [12], [31], [19] 60°C for 30 minutes [18]. The application of formic acid at the lower concentration but higher temperature and shorter time was reported by Valianou *et al.* Where the mixture of formic acid: methanol: water (2:1:1, v/v/v) at 100°C for 15 minutes was used [15].

The red dye was extracted from the wool samples dyed with madder using formic acid in different mixtures: HCOOH: MeOH (5:95, v/v) and HCOOH: MeOH: W (2:1,1, v/v/v) at 92°C for 15 minutes, including ultrasonication. The extracted dyes were studied using CE (**Figure 5.3**). When a mixture of formic acid and methanol; HCOOH: MeOH (5:95, v/v) was used (run a), more alizarin was extracted to compare to a mixture of formic acid and methanol with distilled water; HCOOH: MeOH: W (2:1,1, v/v/v) (run b and c). The same sample used in run b was injected in

run c while the PMT setting was changed from 1000 V to 1200 V. The comparison of these 2 runs showed that the dye extract in run b was reproducible and spiking showed that peak 1 belongs to alizarin.

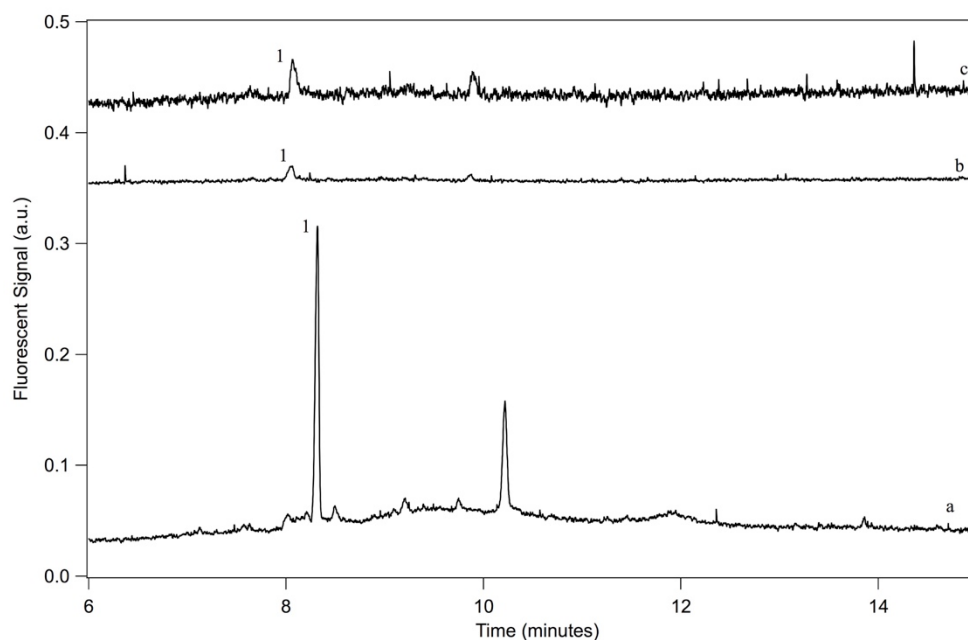


Figure 5.3 Electropherograms of dye extracted from the wool dyed with madder, using HCOOH (a) HCOOH: MeOH (5:95, v/v), (b and c) HCOOH: MeOH: W (2:1:1, v/v/v). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

The amount of the alizarin extracted using different mixtures of formic acid can be explained by HSP values of the solvent. The corresponding R_a values of alizarin versus HCOOH: MeOH (5:95, v/v), HCOOH: MeOH: W (2:1:1, v/v/v) are shown in **Table 5.4**. It can be concluded that the mixture of formic acid and methanol with lower R_a value, 11.8 MP^{1/2} should dissolve alizarin more than the mixture of formic acid, methanol and distilled water with R_a value 12.6 MP^{1/2}. The CE results are in agreement with these values.

Methanol extraction of wool samples dyed with madder

The application of the mixture of methanol and distilled water (1:1, v/v) was reported by Huang *et al.* [32]. The solvent was used to extract synthetic dyes from textiles. The solvent was used under 100°C for unspecified time. In this study for the first time alizarin in the wool sample dyed with madder was extracted using a mixture of methanol and distilled water, at 92°C for 30 minutes, including ultrasonication. **Figure 5.4** shows the result (run a).

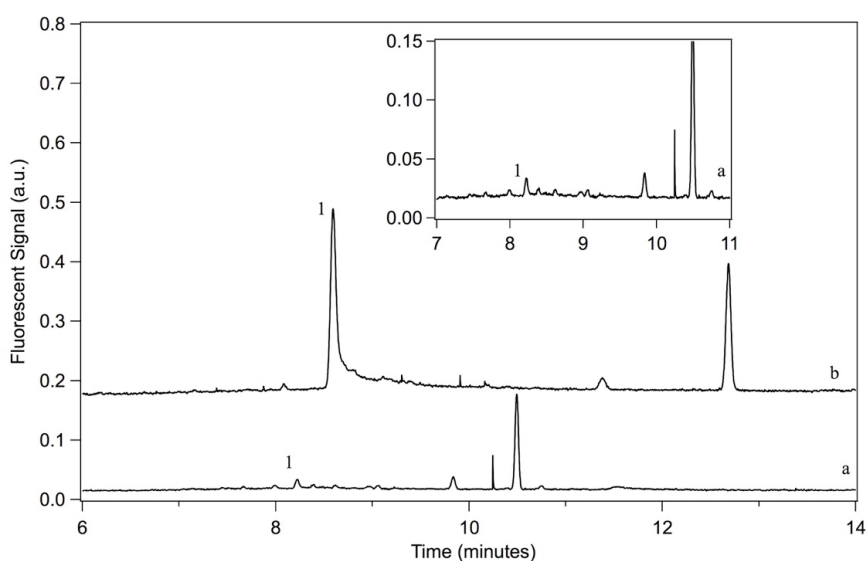


Figure 5.4 Electropherograms of (a) dye extract from the wool dyed with madder using methanol: distilled water (1:1, v/v), (b) the sample from run a was spiked with alizarin. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

In order to identify the small peak eluted around 8 minute, the same sample used in run 'a' was spiked with alizarin and it confirmed that the peak belongs to alizarin.

Pyridine, DMSO and DMF extraction of wool samples dyed with madder

Manhita *et al.* reported the application of different solvents including pyridine, and DMF for extraction of dyes from textile samples belonging to the 18th century. The extraction was performed

at 100°C for 10 minutes [19]. The application of DMSO at 100°C for 10 minutes or as a mixture with HCl at 80°C for 5 minutes were reported [15], [17].

In this study pyridine, DMSO and DMF were used to extract red dye from wool samples dyed with madder. The extraction parameter for these 3 solvents were the same (92°C, for 30 minutes, using ultrasonic wave). The efficiency of the extraction methods was studied by CE. The results from these studies are given in **Figure 5.5**.

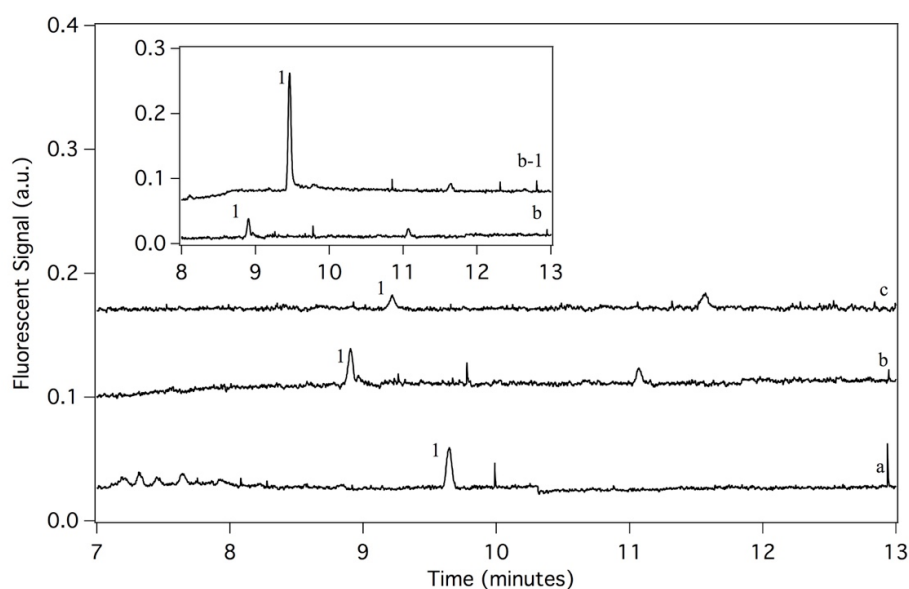


Figure 5.5 Electropherograms of dye extract from madder, using (a) DMF, (b) DMSO, (c) pyridine, (b-1) run b was spiked with alizarin (peak 1). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

The comparison of the electropherograms shows that DMF (run a) and DMSO (run b) could extract a greater amount of alizarin than pyridine (run c). These results are in accordance with the observation of choosing the solvent for extraction. By using Hansen solubility parameters, the extraction efficiency in different solvents can be explained. The corresponding R_a value of alizarin versus DMF, DMSO and pyridine are shown in **Table 5.4**. It can be concluded that DMF with

smallest R_a value, $13.1 \text{ MP}^{1/2}$ should dissolve alizarin more than DMSO and pyridine with R_a values as 13.8 and $16.8 \text{ MP}^{1/2}$, respectively. All these calculated values are in agreement with data from experiment.

5.5.5 Extraction efficiency of weld dyed samples using CE-LIF

Hydrochloric acid (HCl) extraction of wool samples dyed with weld

According to the literature, HCl is not an appropriate solvent to extract yellow dyes since most of the yellow dyes have the glycones and aglycones colouring matters and strong acid hydrolyses the glycosidic bonds [12], [13], [15], [33]. Hydrochloric acid has been diluted with water or water and alcohol to extract yellow dyes. When a solution of 2 M HCl was used to extract the different dye components from Indian textiles and TLC was performed on the extract dye, the yellow dyes were not detected [29]. In terms of application of a mixture of HCl with alcohols, It was reported that (HCl 3M: MeOH, 1:1, v/v) at 100°C for 15 minutes could extract dyes from textiles belonging to the 18th century and luteolin and apigenin could be identified in cream, mustard and green samples by performing HPLC-DAD [34]. Moreover, the mixture of 3 M HCl with ethanol (1:1, v/v) was used to extract yellow dyes. For example, in one study the extraction was performed at 100°C for 15 minutes on the Coptic textiles belonging to the 8th century and luteolin and apigenin were identified in the orange sample, and luteolin in the green one [7]. Moreover, extracted dyes from Coptic textiles by a mixture of 3 M HCl with ethanol (1:1, v/v) were analysed by HPLC-FLD and CE-DAD. It was reported that luteolin and apigenin, and luteolin were identified in yellow and orange

samples, respectively by running CE- DAD, while HPLC-FLD could not identify those dyes [8], [9].

Zhang *et al.* reported the application of methanolic hydrochloric acid in higher concentration HCl 37%: MeOH: W (2,1,1, v/v/v) at different conditions for extraction of yellow dyes. The extraction of dyes from a wool sample dyed with weld using methanolic hydrochloric acid at 100°C for 10 minutes, formic acid and EDTA 0.001M: ACN: MeOH, was compared by performing LC-DAD. Luteolin was the only dye component that could be extracted using different solvents [12]. Peggi *et al.* [13] and Valianou *et al.* [15] studied the extraction efficiency of the wool samples dyed with weld using HCl 37%: MeOH: W (2,1,1, v/v/v) at 100°C introduced by Zhang *et al.* [12] at longer time. They showed that increasing the extraction time from 10 to 15 minutes did not change the extraction results [13], [15]. In these 2 studies by performing LC-DAD the extract dyes were identified as luteolin, apigenin and chrysoeriol (3'-methoxyluteolin).

In this work, 3 M HCl at 60°C for 10 minutes, HCl 3M: EtOH (1:1, v/v) at 92°C for 10 minutes, including ultrasonication, and HCl 37%: MeOH: W (2:1,1, v/v/v) at 60°C for 10 minutes were chosen for extraction from the wool samples dyed with weld. The extracted dyes were studied using CE under separation conditions using 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22kV. The results were in agreement with those of other research groups and only the aglycones of luteolin and apigenin were detected. The trend of extraction efficiency of luteolin and apigenin was as follows:

HCl 3M: EtOH (1:1, v/v) > HCl 37%: MeOH: W (2:1,1, v/v/v) > HCl 3M.

The comparison between the results of extraction of samples dyed with madder and weld using HCl, HCl 3M: EtOH (1:1, v/v) extractions proved to be the most efficient methods for extracting,

alizarin, luteolin and apigenin in the samples. The comparison between the migration time of extracted dyes with the mixture of standard solution of dyes, shows that extracted dyes eluted later. The delay observed was likely due to the extraction of unwanted residue from wool samples by HCl.

Formic acid (HCOOH) extraction of wool samples dyed with weld

The mixture of formic acid and methanol (5:95, v/v) has been used as a favorable solvent for extraction of yellow dyes [12], [15], [31], [19], [18]. The extraction conditions were varied, it was used at lower temperature 40°C for 30 minutes or higher temperatures 60°C for 30 minutes [18]. Also, the mixture of formic acid: methanol: water (2:1:1, v/v/v) was used at 100°C for 15 minutes [15]. Zhang *et al.* believed increasing the temperature of extraction from 40 to 60 °C causes the hydrolysis of the glycosidic bond, while the results from [18] and [15] were not in agreement with this conclusion. However, in this work the extraction was performed at high temperatures (60 and 92°C) and 7-*O*-glycosidic of luteolin and apigenin were detected.

Based on the evaluation of the conditions applied for extraction of dyes from the weld dyed wool samples, 2 methods were preferred: HCOOH: MeOH (5:95, v/v) at 60°C for 30 minutes and HCOOH: MeOH: W (2:1:1, v/v/v) at 92°C for 15 minutes, including ultrasonication. The extraction efficiency was evaluated by CE –LIF and separation results are given in **Figure 5. 6**. The comparison of electropherograms showed that the mixture of HCOOH: MeOH (5:95, v/v) (run a) could extract 4 flavones: lu-7-*O*-glu (peak 1), ap-7-*O*-glu (peak 2), apigenin (peak 3) and luteolin (peak 4). The mixture of HCOOH: MeOH: W (run b) extracted lu-7-*O*-glu (peak 1), and

ap-7-*O*-glu (peak 2), the same amount as formic acid and methanol did. This solvent could extract more luteolin (run b, peak 3) compared to the mixture of formic acid and methanol (run a, peak 4). Apigenin was not extracted using this method.

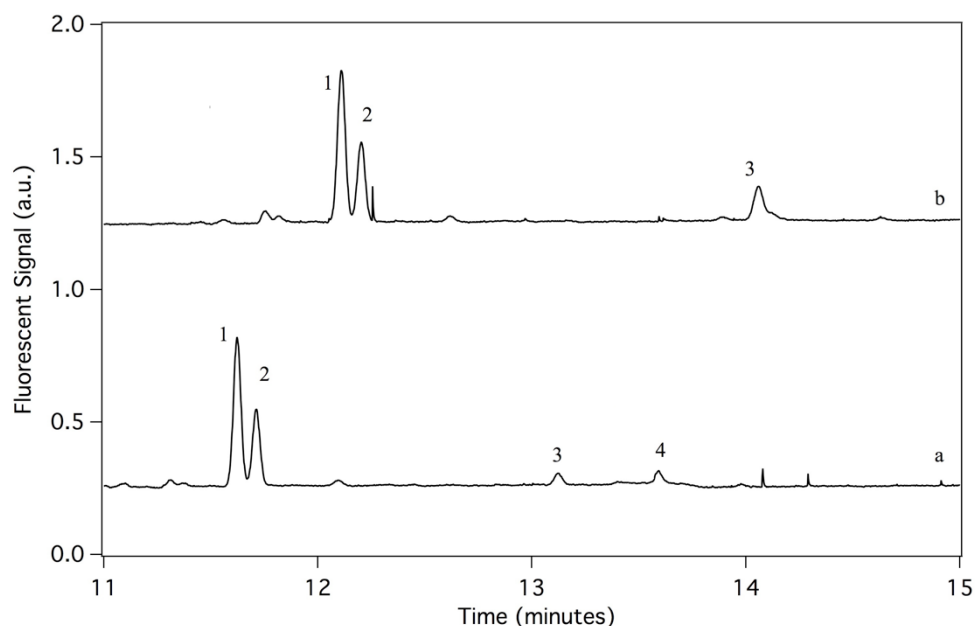


Figure 5.6 Electropherograms of dye extract from the wool dyed with weld, using HCOOH: (a) HCOOH: MeOH (5:95, v/v) lu-7-*O*-glu (peak 1), ap-7-*O*-glu (peak 2), apigenin (peak 3) and luteolin (peak 4), (b) HCOOH: MeOH: W (2:1:1, v/v/v) lu-7-*O*-glu (peak 1), ap-7-*O*-glu (peak 2), and luteolin (peak 3). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

The amount of the flavones extracted using different mixtures of formic acid can be explained by HSP values of the solvent. The corresponding R_a values of luteolin and apigenin versus HCOOH: MeOH (5:95, v/v), and HCOOH: MeOH: W (2:1:1, v/v/v) are shown in **Table 5.4**. The R_a values of luteolin and apigenin in a mixture of HCOOH: MeOH (5:95, v/v) 18.1, and 16 MP^{1/2} were lower than those in the mixture of HCOOH: MeOH: W (2:1:1, v/v/v) 20.4 and 18.3 MP^{1/2}. A reduced R_a value should show more solubility; however, the CE results did not confirm this

hypothesis. It is possible that performing the extractions at 2 different temperatures could affect the HSP values of solvent mixtures and in return have affected the R_a values.

According to the literature, the colouring matter in the sample dyed with weld was varied, which may be due to the extraction method or the source of the weld. Peggie *et al.* [13] and Valour *et al.* [15] used HPLC-DAD to detect luteolin, apigenin and chrysoeriol as the colouring matters extracted using methanolic HCl from the samples dyed with weld [13], [15]. However, by using the milder extraction solvent; methanolic formic acid for weld dyed samples and running the same detection technique, both glycones and aglycones colouring matters in weld were detected: lu-7-*O*-glu and apigenin-*O*-glucoside as well as luteolin, apigenin, chrysoeriol [15]. Furthermore, Sanyova *et al.* [14] reported that both glycosides and aglycone flavones were extracted by using 4 M HF as the mild extraction method. The colourant matters from the samples dyed with weld were detected as: luteolin-3,7'-di-*O*- glucoside, lu-7-*O*-glu, apigenin-*O*- glucoside, luteolin and apigenin [14]. Moreover, Petroviciu *et al.* used methanolic hydrochloric acid for the extraction and by performing HPLC-DAD on the extract only chrysoeriol was detected [16]. Zhang *et al.* performed the same extraction technique on the extract obtained using methanolic formic acid and the only dye among other eluted components assigned was luteolin [12].

Methanol extraction of wool samples dyed with weld

Huang *et al.* also used methanol and water at 100°C for extraction of synthetic dyes from textiles [32]. In our work for the first time the colouring matters of weld dyed wool sample were extracted using a mixture of methanol and distilled water (1:1, v/v) at 92°C for 30 minutes, including ultrasonication.

The CE study of these samples (**Figure 5.7**) showed that methanol: water (1:1, v/v) could extract 4 yellow flavones (run a). The dyes were assigned by spiking with standard solutions (run b) as: lu-7-*O*-glu (peak 1), ap-7-*O*-glu (peak 2), apigenin (peak 3) and luteolin (peak 4).

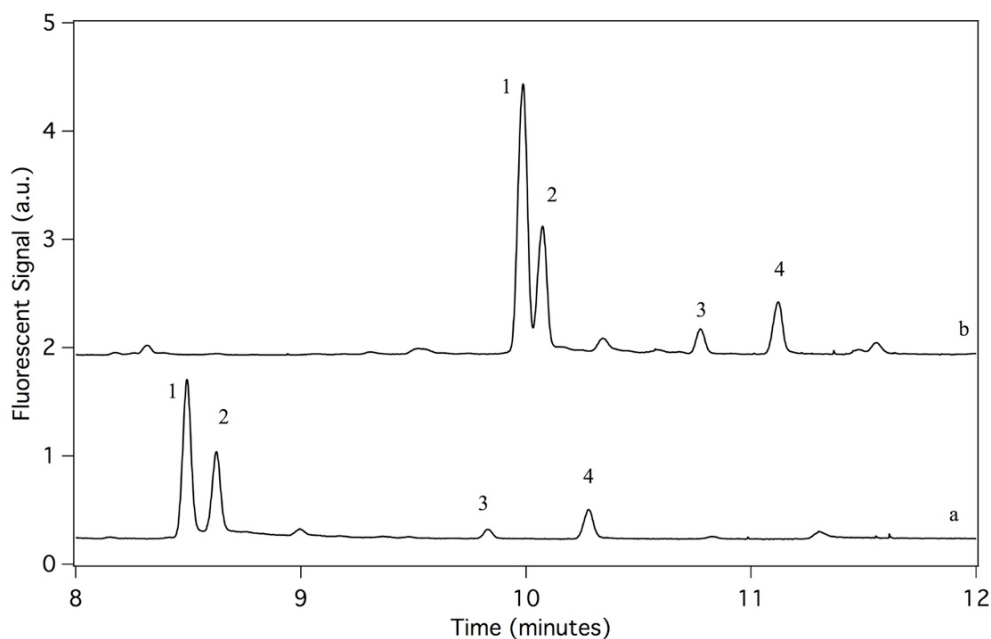


Figure 5.7 Electropherograms of dye extract from the wool samples dyed with weld, using **(a)** MeOH : distilled water (1:1, v/v), **(b)** the extract solution was spiked with standard dyes solution, and the peaks were assigned as lu-7-*O*-glu (peak 1), ap-7-*O*-glu (peak 2), apigenin (peak 3) and luteolin (peak 4). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

According to **Table 5.4** higher R_a values were observed for luteolin and apigenin in the mixture of methanol and water compared with methanol. Since higher temperatures could be applied to the mixture of methanol and water, more dyes were extracted.

Pyridine, DMSO and DMF extraction of wool samples dyed with weld

Pyridine and DMF were used at 100°C for 10 minutes for extraction of the 18th century textile samples [19]. Valiar *et al.* has reported the use of DMSO at 100°C for unspecified time [15]. In this study pyridine, DMSO and DMF were used to extract flavone dyes from the weld dyed wool samples at 92°C, for 30 minutes, using ultrasonication. The efficiency of the extraction methods was studied by CE. The results from these studies are given in **Figure 5.8**.

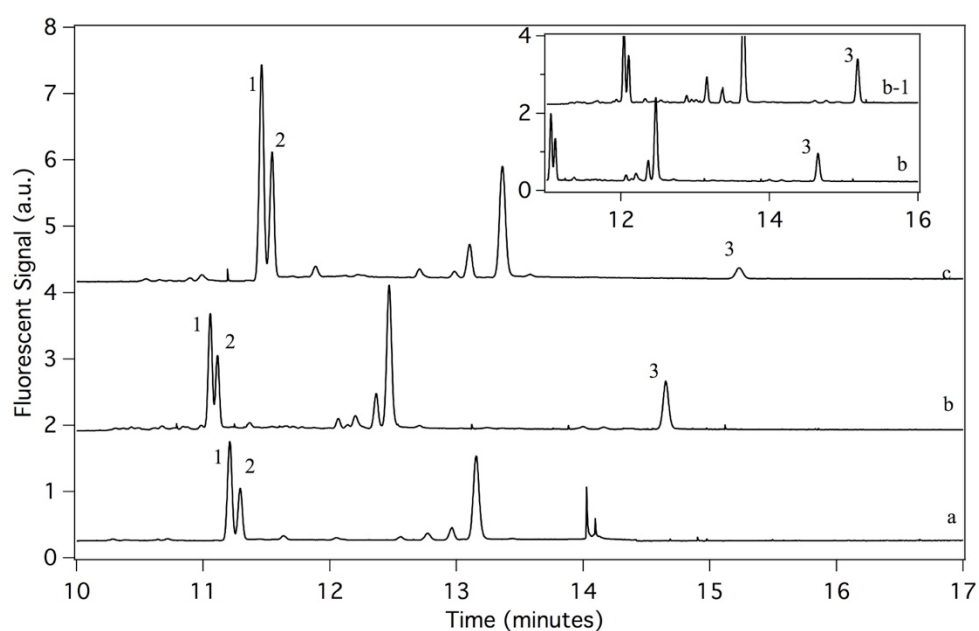


Figure 5.8 Electropherograms of extracted dyes from the wool samples dyed with weld, using (a) pyridine, (b) DMSO and (c) DMF. Embedded picture (b-1) is the spiking of run (b) with luteolin (peak 3). Spiking showed that. The peaks were eluted as lu-7-*O*-glu (peak 1), ap-7-*O*-glu (peak 2) and luteolin (peak 3). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

This study showed that none of these solvents were able to extract apigenin. Pyridine (run a) was able to extract only 2 dyes: lu-7-*O*-glu (peak 1) and ap-7-*O*-glu (peak 2), while DMSO and DMF could extract 3 dyes: lu-7-*O*-glu (peak 1) and ap-7-*O*-glu (peak 2), and luteolin (peak 3) (run b and c, respectively). DMF was able to extract more amounts of lu-7-*O*-glu (peak 1) and ap-7-*O*-

glu (peak 2), in comparison with DMSO and pyridine. However, luteolin (peak 3) was extracted more with DMSO which is in contradiction to the R_a values given in **Table 5.4**. According to **Table 5.4** pyridine should dissolve more amount of luteolin in comparison with other solvents.

The comparison between the electropherograms of extract from the samples dyed with madder and weld showed that most solvents could extract alizarin to the same extent. In terms of flavones, the mixture of HCl with ethanol, formic acid and methanol as well as methanol and water could extract all 4 colouring agents in weld while other solvents could extract 2-3 of the flavones. Therefore, to extract dyes from the case study samples methanol and water (1:1, v/v) was selected due to low cost, environmentally friendly, and low boiling point compared to other organic solvents. This property enables the extract to dry at room temperature in a shorter period of time. In following, the results from the separation and identification of colouring agents in the case study samples with CE-LIF are given.

5.6 Case studies

The dyes were extracted from following authentic samples using a methanol and water mixture.

The historical samples for this research were: yellow, red, green and brown threads from a piece of hand woven carpet (100-year-old). Yellow and red silk threads from a Chinese robe belonging to Chinese dynasty (1700 AD) afforded by Alberta Museum (Edmonton). Yellow and red wool threads from clothing of the “Salt Man” (600 BC) provided by Research Center for Conservation

of Cultural Relics (RCCCR), Tehran. And a piece of cloth with yellow, red and gold threads from Safavid time (1700 AD) was kindly provided by the National Museum of Iran. A red thread of a table cloth from the 20th century was afforded by Winnipeg Art Gallery. Moreover, the colourants in paper dyed with henna, clove, cochineal, madder were investigated using CE-LIF. Also, the extraction colourants in some plants including: onion skin, henna, clove, madder, weld, Persian burries were explored.

5.6.1 Persian Carpet

A piece of 100-year-old hand woven colourful Persian carpet was provided from Iran by a carpet merchant (**Figure 5.9**).



Figure 5.9 A piece of 100-year-old hand woven colourful Persian carpet

Different threads were chosen to study the dye component using CE-LIF. The results of CE separation (run a) and spiking with standard solutions (run b) of red, yellow, brown and green threads are presented in **Figures 5.10 to 5.13**.

Persian Carpet: Red wool

The extract from 1mg of red thread was dissolved in borate buffer and CE separation was performed on the sample, **Figure 5.10**, run 'a'. Spiking the same solution with $1\mu\text{g ml}^{-1}$ alizarin standard solution confirmed that peak 1 belonged to alizarin. It could be concluded that the thread was dyed with madder plant.

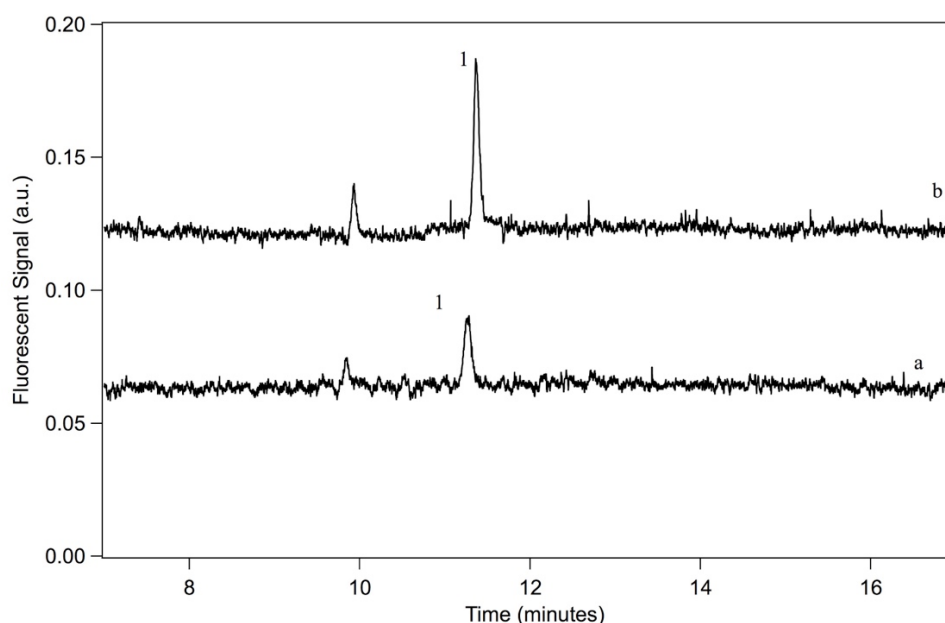


Figure 5.10 Electropherograms of **(a)** dye extract from the red wool from the carpet using methanol: distilled water (1:1, v/v), **(b)** the sample from run a was spiked with alizarin. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

Persian Carpet: Yellow wool

The extract from 1mg of yellow thread was dissolved in borate buffer and CE separation was performed on the sample, **Figure 5.11**, run 'a'. The same solution was spiked with 0.1 and 0.2 $\mu\text{g ml}^{-1}$ apigenin and luteolin (run b). The comparison between 2 runs confirmed that peak 1 and 2 belonged to apigenin and luteolin, respectively. The CE separation of the spiked sample with luteolin-7-*O*-glucuronide and apigenin-7-*O*-glucuronide confirmed their presence. It could be concluded that the yellow thread was dyed with weld plant.

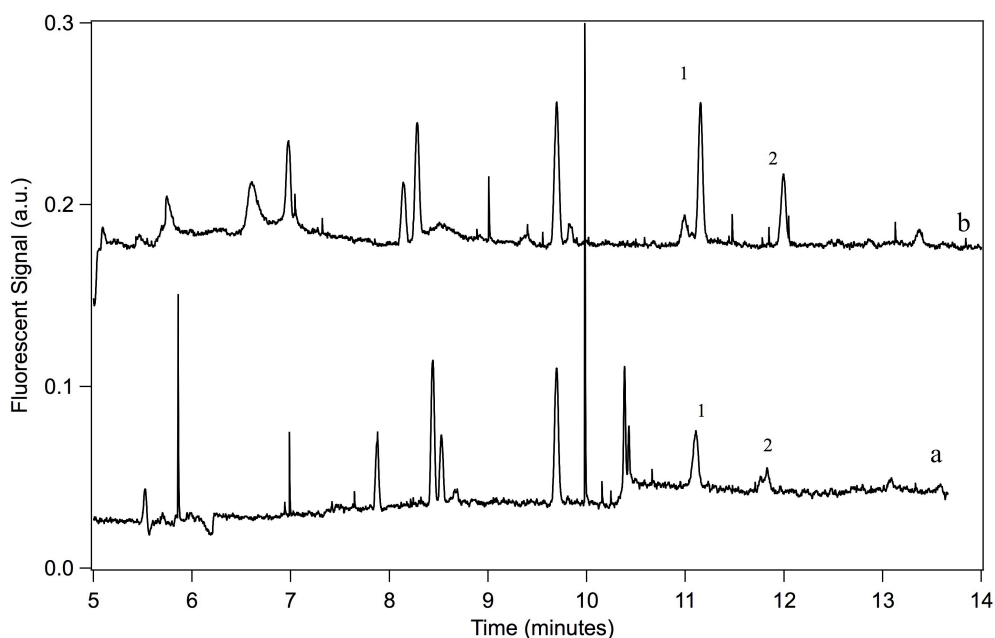


Figure 5.11 Electropherograms of **(a)** dye extract from the yellow wool from the carpet using methanol: distilled water (1:1, v/v), **(b)** the sample from run a was spiked with apigenin and luteolin. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

Persian Carpet: Brown wool

The extract from 1mg brown thread was dissolved in borate buffer and CE separation was performed on the sample, **Figure 5.12**, run 'a'. The same solution was spiked with $2 \mu\text{g ml}^{-1}$ alizarin (run b). It was noticed that only peak 1 in run 'a' showed an increment after spiking and it was assigned as alizarin. Spiking with flavonoids did not confirm the presence of yellow dyes. The brown colour can be made by mixing red and blue dyes. Also, madder with iron mordanting induces brown colour [35]. Performing HPLC-DAD on the extract from the brown sample from Coptic textile belonging to the 4th century could detect following dyes: indigo, purpurin, alizarin, and indigotin. Moreover, the same study on the sample from the 6th century only revealed the presence of alizarin and purpurin [7].

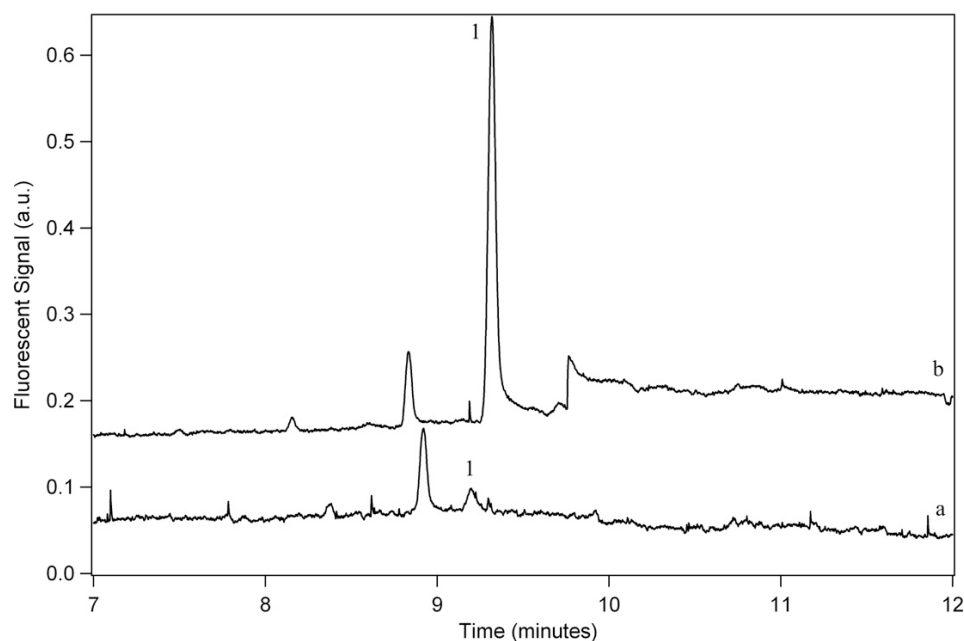


Figure 5.12 Electropherograms of (a) dye extract from the brown wool from the carpet using methanol: distilled water (1:1, v/v), (b) the sample from run a was spiked with alizarin. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

Persian Carpet: Green wool

The extract from 1mg of green thread was dissolved in borate buffer and CE separation was performed on the sample, **Figure 5.13**, run 'a'. The same solution was spiked with $0.5 \mu\text{g ml}^{-1}$ alizarin and luteolin standard solutions (run b). The comparison of the results confirmed that peak 1 and 2 belonged to alizarin and luteolin, respectively. The green colour is mostly achieved by over dyeing a weld dyed yarn with indigo [10]. Also, the presence of red dye in the green colour has been reported by Orska-Gawryś. The analysis of green samples from the Coptic textiles belonging to the 7-9th and 6th centuries, indicated the presence of indigo, indigotin, alizarin and luteolin. Furthermore, a study on another green sample from 7-9th centuries could detect indigo, indigotin and alizarin [7].

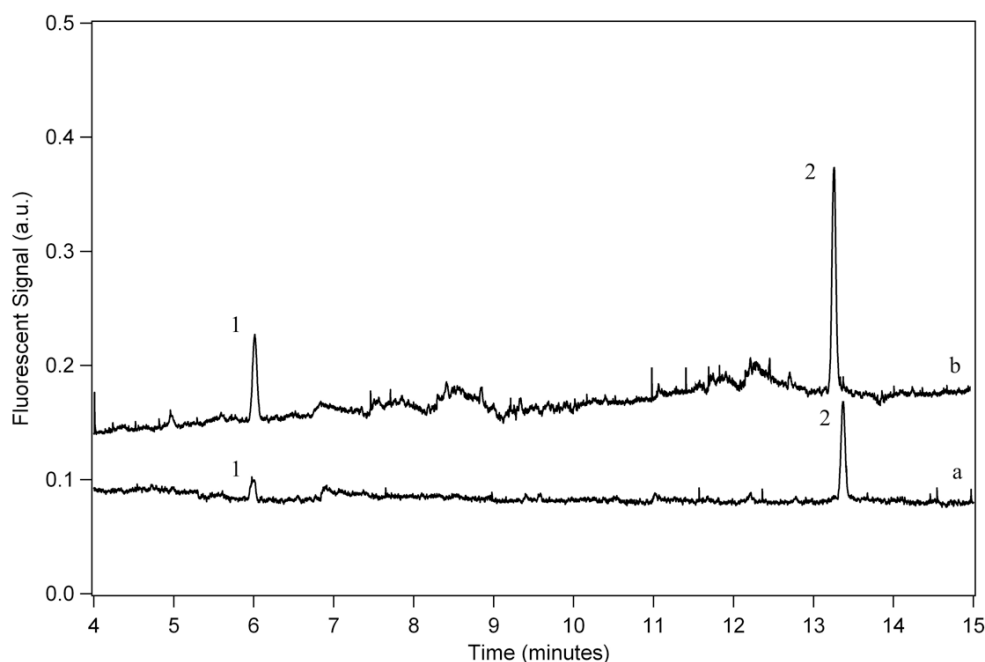


Figure 5.13 Electropherograms of (a) dye extract from the green wool from the carpet using methanol: distilled water (1:1, v/v) (b) the sample from run a was spiked with a lu-7-*O*-glu and luteolin. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

It can be concluded that weld and madder have been used with other dyes like indigo and woad to achieve green colour [36]. The CE separation confirmed that the source of yellow colourants to dye yellow and green samples were different.

5.6.2 Imperial Chinese Robe Samples

Two minute yellow and one small red thread samples belonging to an embroidered yellow silk imperial twelve dragon robe (1736-1795), as well as a minute yellow thread from imperial yellow semiformal (1775-1795) were provided by the Royal Alberta Museum. They are known as: 1: 2005.5.375 Embroidered yellow silk imperial twelve dragon robe (1736-1795): yellow sample from under in-seam (0.75mg); 2: 2005.5.375 Embroidered yellow silk imperial twelve dragon robe

(1736-1795): yellow sample from loose end (0.31mg), 3: 2005.5.375 Embroidered yellow silk imperial twelve dragon robe (1736-1795): red embroidery thread sample from loose end (0.28mg), and 4: 2005.5.9 imperial yellow semiformal (1775-1795): yellow sample from R.R. lower in seam CL (1mg used). Less than 1mg of these samples were used to extract dyes using methanol and water as explained in the previous section. The extract was dissolved in DMSO and the preparation was followed according to the procedure explained in CE sample preparation. The results of CE separation are given in **Figure 5.14 to 5.17**. **Figure 5.14** run 'a' shows the separation of components in the yellow sample from embroidered yellow silk imperial twelve dragon robe (yellow 1).

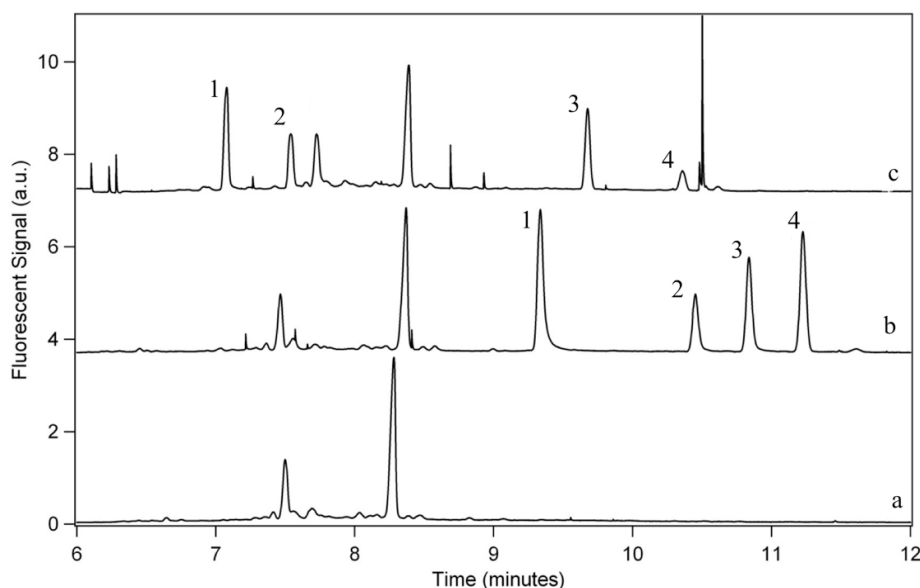


Figure 5.14 Electropherograms of **(a)** dye extract from embroidered yellow silk imperial twelve dragon robe (yellow 1) using methanol: distilled water (1:1, v/v), **(b)** the same sample spiked with $1 \mu\text{g ml}^{-1}$ of standard solutions as assigned peaks: kaempferol (1), myricetin (2), quercetin(3), and morin (4), **(c)** the fresh extract spiked with $3 \mu\text{g ml}^{-1}$ lu-7-*O*-glu (1), $1 \mu\text{g ml}^{-1}$ ap-7-*O*-glu (2), apigenin (3) and luteolin (4). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

In order to assign the peaks a fresh sample of the extract was used for spiking with flavonols (run b) and flavones (run c). None of the dyes could be assigned using the 8 common dyes in our data bank.

Figure 5.15 run a shows CE separation of another yellow sample from the same robe.

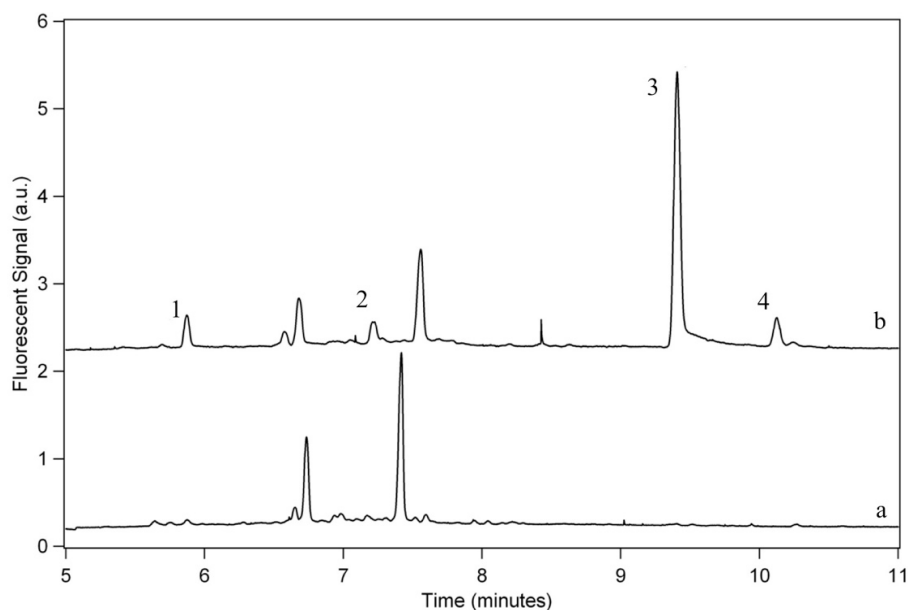


Figure 5.15 Electropherograms of **(a)** dye extract from embroidered yellow silk imperial twelve dragon robe (yellow 2) using methanol: distilled water (1:1, v/v), **(b)** the same sample spiked with standard solutions as assigned: peak (1) $\mu\text{g ml}^{-1}$ lu-7-*O*-glu, peak (2) $1 \mu\text{g ml}^{-1}$ ap-7-*O*-glu, peak (3) $0.1 \mu\text{g ml}^{-1}$ apigenin and peak (4) $1 \mu\text{g ml}^{-1}$ luteolin. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

Figure 5.15 run 'b' shows the spiking the fresh solution of yellow dye (run a) with standard solutions of flavones and none of the peaks could be identified.

CE was performed on the extract from the third yellow sample, imperial yellow semiformal (1775-1795) but the same as other yellow samples the spiking with standards did not help to identify the peaks. The CE profile of these 3 yellow samples are presented in **Figure 5.16**. The comparison between the CE runs shows that these samples were dyed with the same source of dye.

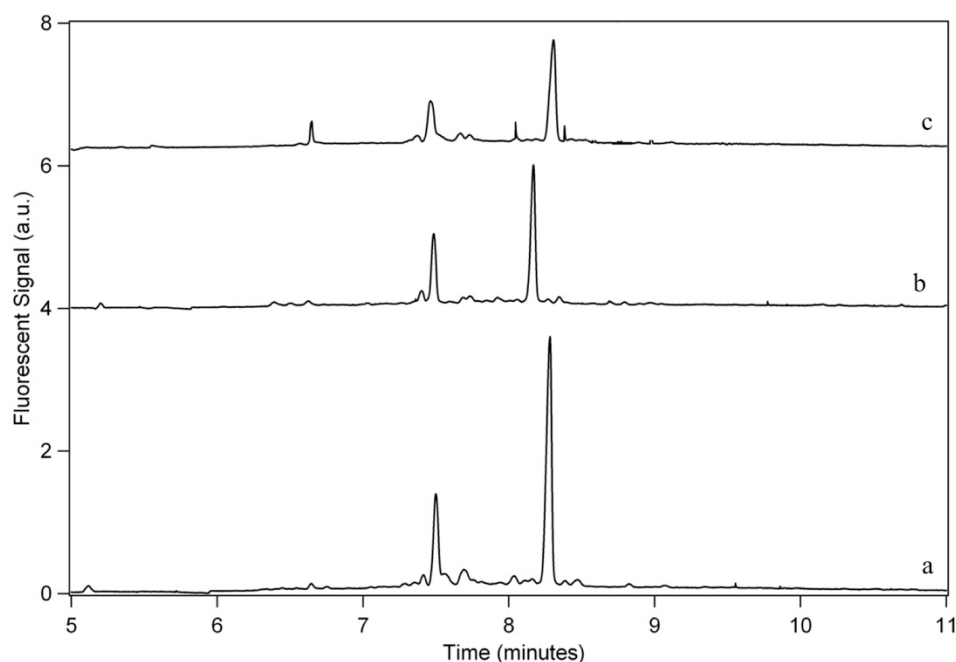


Figure 5.16 Electropherograms of dye extract from embroidered yellow silk imperial twelve dragon robe: **(a)** sample yellow 1, **(b)** sample yellow 2 and **(c)** imperial yellow semiformal sample yellow 3; using methanol: distilled water (1:1, v/v). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

Figure 5.17 run a shows the separation of components in the red sample from embroidered silk imperial twelve dragon robe. In order to assign the peaks a fresh sample of the extract was used for spiking with red standard solutions (run b). The spiking confirmed the presence of alizarin in the extract. Since the peak of carmine (peak 3) was not a match, it could be concluded that the animal base dyes (cochineal) had not been used for dyeing the red threads.

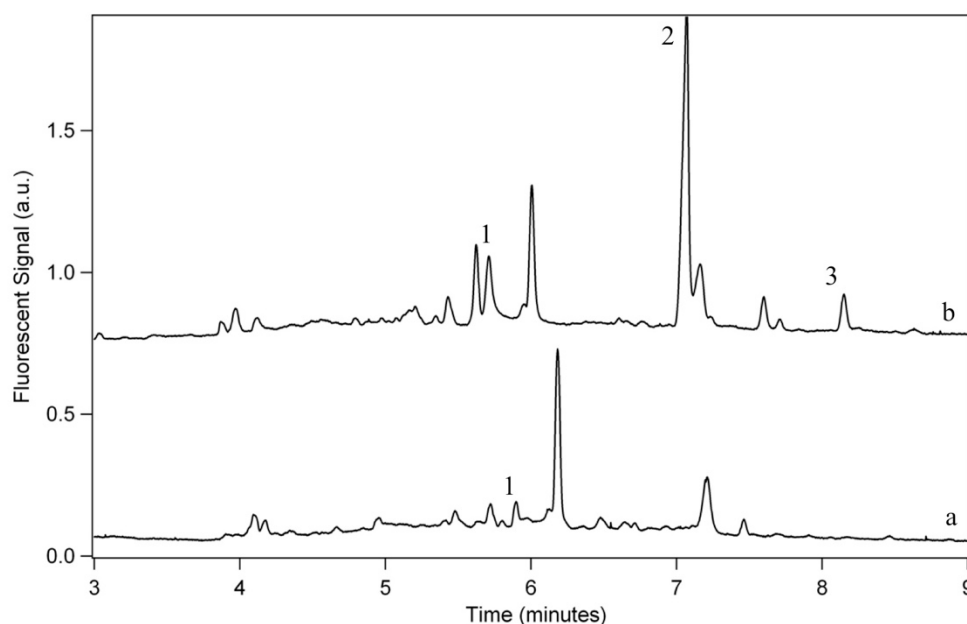


Figure 5.17 Electropherograms of **(a)** red dye extract from embroidered silk imperial twelve dragon robe using methanol: distilled water (1:1, v/v); peak 1 assigned as alizarin, **(b)** the same sample spiked with $2 \mu\text{g ml}^{-1}$ standard solution of alizarin (peak 1) and $5 \mu\text{g ml}^{-1}$ solutions of emodin (peak 2) and carmine (peak 3), respectively. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

Other research groups studied different Chinese items belonging to the same time span. This showed that the source of the dye used in the objects were different. For example, the dyes detected in the pink colour of the 18th century wall paper were carminic acid, laccaic acid A, indigotin, and indirubin [14]. While the red dyes in a pair of shoes and the head band were identified as carthamin the colourant in safflower and brazilin the colourant in brazilwood. The yellow dyes in these sample were analysed and reported as berberine, palmatin the main colourants found in Asian berberis and rutin (quercetin-3-o-glucoside), quercetin the main colourants in Chinese yellow berries [31].

5.6.3 The textile samples from “Salt Man”

Six human mummified remains were found in a Chehrabad (Douzlakh) salt mine located in Zanjan province, northwest of Iran, during the time span of 1993 to 2008. These remains were designated “Salt Men” numbered one to 6 [37], [38]. In this study the samples were taken from the clothing of the first “Salt Man” who was found in 1993 in a mine excavation. The ^{14}C studies showed that he lived during the early Sassanid dynasty (224-651 AD) [39]. His clothing and possessions suggest that he belonged to a high rank of society [40].

Red, yellow and green threads belonging to a multi-coloured sample 10141, a piece of garment, as well as minute red thread from sample 10050 a fragment of belt were provided by The National Museum of Iran located in Tehran. It was reported previously that these samples were mostly made of wool and minor amount of goat hair [35].

Less than 1mg of these samples was used to extract dyes using methanol and water as explained in the previous section. The extract was dissolved in DMSO and procedure explained in CE sample preparation was used. The results of CE separation are given in **Figures 5.18 to 5.21**. The CE separations of the red samples 10141 and 10050 are presented as run ‘a’ in **Figure 5.18 and 5.19**, respectively. In order to assign the peaks, the solutions used in run ‘a’ were spiked with a $1\text{ }\mu\text{g ml}^{-1}$ standard solution of alizarin (run ‘b’ in both Figures) and it was concluded that the peak eluting at 8:30 minutes belonged to alizarin. The results of this study were in agreement with the findings reported by other research groups who worked on red threads from samples 10141 and 10050 [40]. In a preliminary study the red dyes from samples were extracted using hot HCl, and by performing TLC and IR spectroscopy alizarin was detected and it was concluded that these samples would be dyed using madder [40].

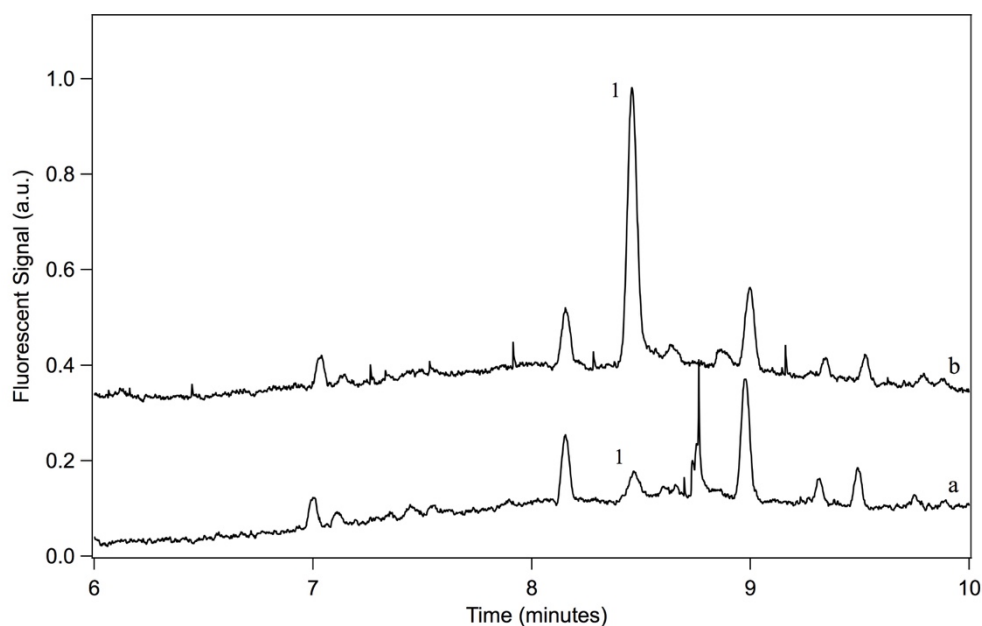


Figure 5.18 Electropherograms of (a) dye extract from red thread from sample 10141 using methanol: distilled water (1:1, v/v), (b) the same sample spiked with 1 $\mu\text{g ml}^{-1}$ of alizarin standard solution. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

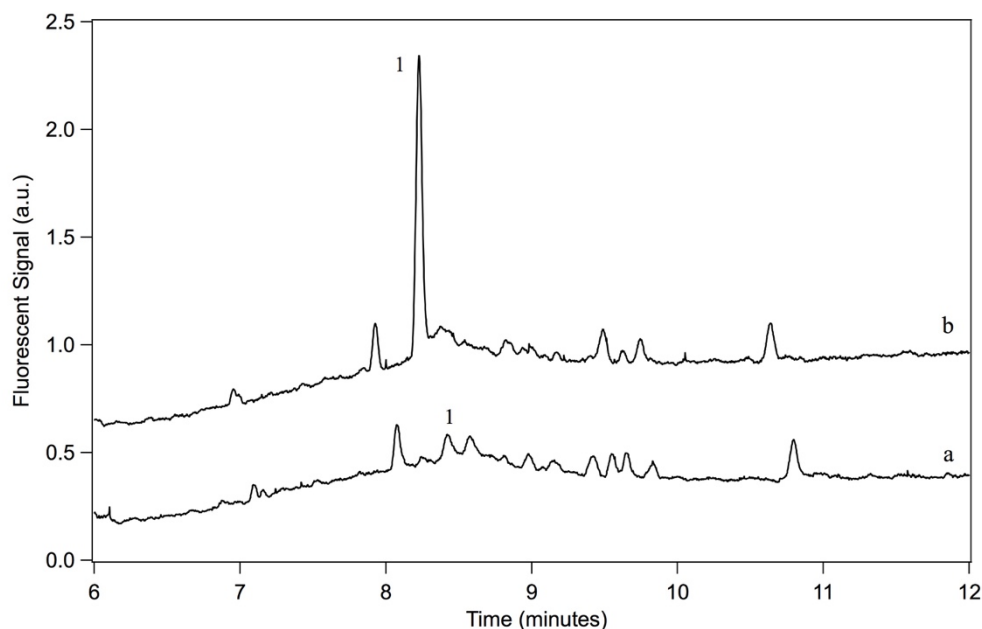


Figure 5.19 Electropherograms of (a) dye extract from red thread from sample 10050 using methanol: distilled water (1:1, v/v), (b) the same sample spiked with 1 $\mu\text{g ml}^{-1}$ of alizarin standard. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

Also, the chemistry department of Boston University and Williamstown Art Conservation Center analysed the same samples and the presence of alizarin and a little amount of purpurin were reported [35].

The CE separation of the yellow sample 10141 is presented as run 'a' in **Figure 5.20**. In order to assign the peaks, the solution used in run 'a' was spiked with standard solutions of $0.2 \mu\text{g ml}^{-1}$ lu-7-*O*-glu, ap-7-*O*-glu, luteolin and $0.01 \mu\text{g ml}^{-1}$ apigenin

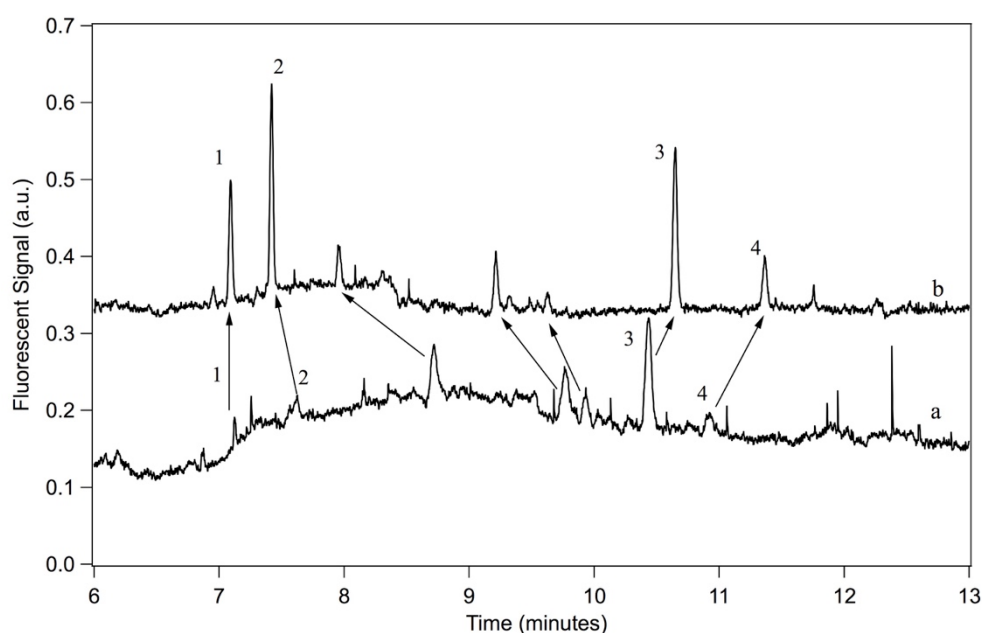


Figure 5.20 Electropherograms of (a) dye extract from yellow thread from sample 10141 using methanol: distilled water (1:1, v/v), (b) the same sample spiked with flavones standard solutions and the peaks were assigned as: lu-7-*O*-glu (peak 1), ap-7-*O*-glu (peak 2), apigenin (peak 3) and luteolin (peak 4). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

The elution order of these dyes is assigned in run 'b' as luteolin-7-*O*-glucoside, ap-7-*O*-glu, apigenin and luteolin. Except apigenin eluted before luteolin; the order of elution was in agreement with the order reported by Sanyova for the samples dyed with weld [14]. From these results, it can

be concluded that the yellow thread was dyed with weld. Also, the findings from performing FT-IR and UV spectroscopy showed that the yellow thread should be dyed by weld [35]. Moreover, the Chemistry Department of Boston University and Williamstown Art Conservation Center analysed the same sample and flavonol sulfate was detected. This dye is one of the rare colourants which was not included in the 200 dyes database. It was concluded the plant used for dyeing should be a local one growing in Zanzibar province [35].

The CE separation of the green sample 10141 is presented as run 'a' in **Figure 5.21**. In order to assign the peaks, a fresh solution as used in run 'a' was spiked with standard solutions of $0.04 \mu\text{g ml}^{-1}$ yellow and red dyes including: lu-7-*O*-glu, ap-7-*O*-glu, luteolin, apigenin and alizarin. Spiking confirmed the presence of a small amount of alizarin in the green sample, as opposed to the other research groups reporting that the green dye in this sample was a mixture of only blue and yellow dyes [35]. Moreover, the findings of the Chemistry Department of Boston University and Williamstown Art Conservation Center showed that the green colour was achieved by mixing flavonoids and indigoids [35]. Our finding regarding alizarin, may be due to the dye pot have been contaminated with madder.

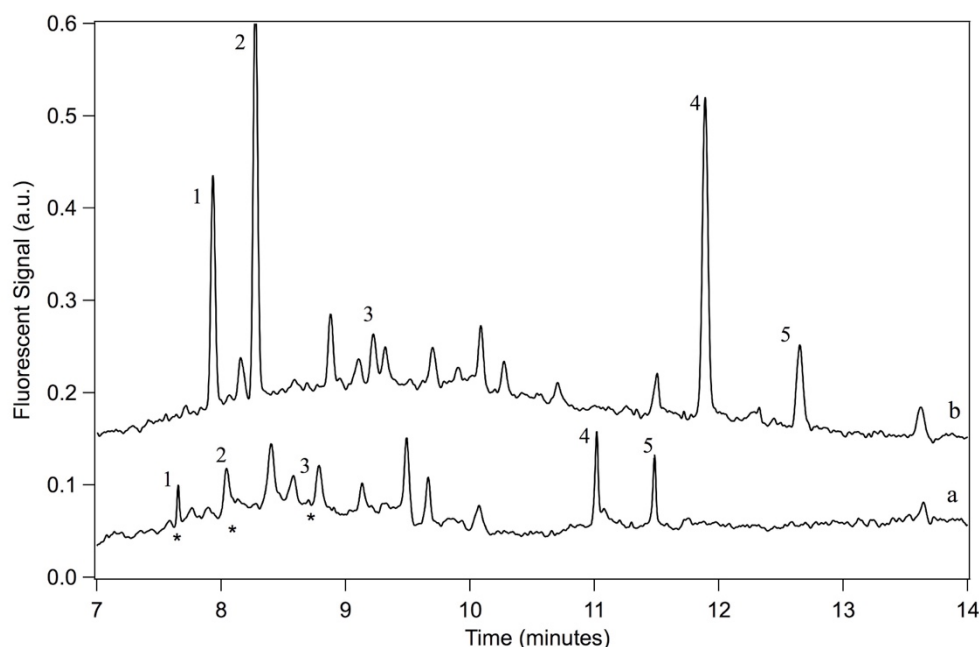


Figure 5.21 Electropherograms of **(a)** dye extract from green thread from sample 10141 using methanol: distilled water (1:1, v/v) , **(b)** the same sample spiked with flavones standard solutions and the peaks were assigned as: lu-7-*O*-glu (peak 1), ap-7-*O*-glu (peak 2) , alizarin (peak 3), apigenin (peak 4) and luteolin (peak 5). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

Moreover, all dyes in the samples under this study were also analysed by Mouri *et al.* The dyes were extracted using pyridine: water: oxalic acid 1.0M (95:95:10) at 100°C for 15 minutes and detected by HPLC-DAD-MS [41]. The results for red samples were in agreement with our findings. The peak belong to alizarin was detected at 50 minute and it was concluded that the samples were dyed by madder [41]. In terms of yellow dyes, this research group had 2 yellow samples from 2 different textile fragments 10050 and 10141. HPLC- DAD was performed on the sample 10050 and the following dyes were detected: ellagic acid, quercetin glucuronide, quercetin 3-o-sulfate, kaempferol 3-o-sulfate, isorhamnetin or tamarixetin 3-o-sulfate, quercetin (3',4 or 7)-o-sulfate, quercetin and alizarin. These dyes are the most common dye components found in the tamarix

plant, the local plant grows near the salt mine. However, the results for yellow sample 10141 (the sample studied in our lab) was not the same as sample 10051, and the following dye components were identified: quercetin 3-o- glucuronide, quercetin 3-o-glucoside, and other 3-o-flavonol glucosides. From this data the conclusion was drawn that these 2 yellow samples had different sources and the source of sample 10041 was unidentified.

Regarding the green sample (10041), it was concluded that it was made of the mixture of blue and yellow dyes. Where the source of yellow dyes was the same as the one used for dying yellow sample 10040 sample and blue source could be possibly woad [41].

5.6.4 Safavid Brocade

A piece of brocade belonging to the 17th century used for Shah-Abbas funeral was provided by The National Museum of Iran located in Tehran. **Figure 5.22** shows the verso (back) and recto (front) of this fragment. As it can be seen the red and blue threads were used in verso and the golden, blue and yellow threads were used in recto. The blue threads were used for writing the verses of Surah al-Nasr and in perpendicular and vertical forms in the wrap



Figure 5.22 The verso and recto of the fragment belonging to the Safavid period.

golden threads, and the yellow-orange threads were used as decoration among the blue calligraphy. The 2 large pieces of this textile are in the National Museum of Iran (Tehran) and Shrine Museum in Qum. These textiles are introduced in the book of Shah Abbas: The Remaking of Iran [42].

Safavid Brocade: Golden thread

The art of using gold threads in Persian textiles goes back to the Achaemenid era (529-550 B.C.) and it reached to its apex in the Sassanid time (226-642 A.D.). This art declined in the early centuries after the Arab invasion. During the Mongol period (1220 A.D.) so many weaving centers were destroyed and the pure gold threads were replaced with yarns covered with gold or silver layers. After the founding of the Safavid dynasty in 1502 A.D. the textile art flourished again and in Shah- Abbas time (17th century), a famous textile complex was established in Isfahan comprising of dyeing, weaving and designing centers, only for export purposes. The gold textiles which are known as *zarbaft* (*zar*= gold, *baft*= weaving) or brocade belonging to the Safavid time are well-known due to the application of high quality silver and gold threads as well as having sophisticated designs with beautiful colours. It is worth noting that in 1700 A.D. for fabrication of metal threads, a manual was used describing: the ratio of silver and gold for production of silver threads, the golden threads using in brocades, and highest quality golden threads. The percentage of Au in these 3 items was defined as 5, 10 and 15 %, respectively [43].

The threads of the fragment were analysed using portable XRF and the red, yellow threads were studied using CE-LIF. The results are given in following. The bright yellow colour of the wrap threads suggested that it was Au. The results from XRF confirmed this thread is made mostly of Au, Ag and has a small amount of Fe and Cu (**Figure 5.23**).

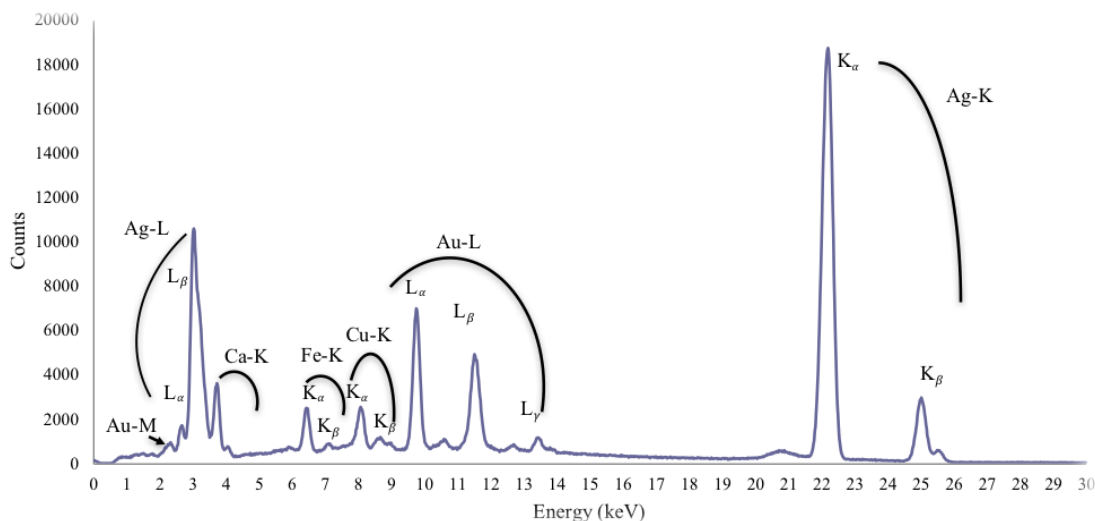


Figure 5.23 The XRF spectrum of the golden thread used in the Safavid brocade belonging to 17th century

The study of a golden thread belonging to the Safavid time by SEM showed the presence of Au, Ag, Hg, S and Cl [43]. However, the XRF spectrum did not show the K, L and M Hg- lines: 80.255, 70.82 (K lines), 11.824 (L line), and 2.281, 2.195 (M lines); as well as K S- lines at 2.3, 2.45. Also, it could be there was an overlap between Au M and Cl K X-ray energies at 2.622, 2.812. Shibayama *et al.* also reported the application of metal threads in the Persian velvet belonging to the 16th – 18th centuries. The SEM analysis revealed that the metal threads were made of silver with a very small amount of copper and most of them were gilded [44].

The yellow and red dyes were extracted from the threads using the procedure explained in previous section and the dyes were dissolved in DMSO following the procedure for sample preparation for CE studies. Results are given in **Figures 5.24** and **5.25**.

Safavid Brocade: Yellow thread

The CE separation of the yellow thread is presented in **Figure 5.24**. In order to assign the peaks, 8 fresh solutions of the yellow extract were spiked with each yellow standard dye solution (the electropherograms are not given) and the comparison of the changes in the peaks helped to assign peaks 1, 2 and 3 as luteolin-7- o- glucoside, apigenin and luteolin, respectively. It can be concluded that weld was used as the source of yellow dye. However, HPLC-PAD study of the yellow and dyes used in the Persian velvet belonging to the 16- 18th A.D. showed that 8 out of 10 yellow pile samples were dyed with larkspur (main colourants: quercetin 3-o-hexoside, and kaempferol 3-o-hexoside) and the rest with luteolin, and apigenin containing dye [44].

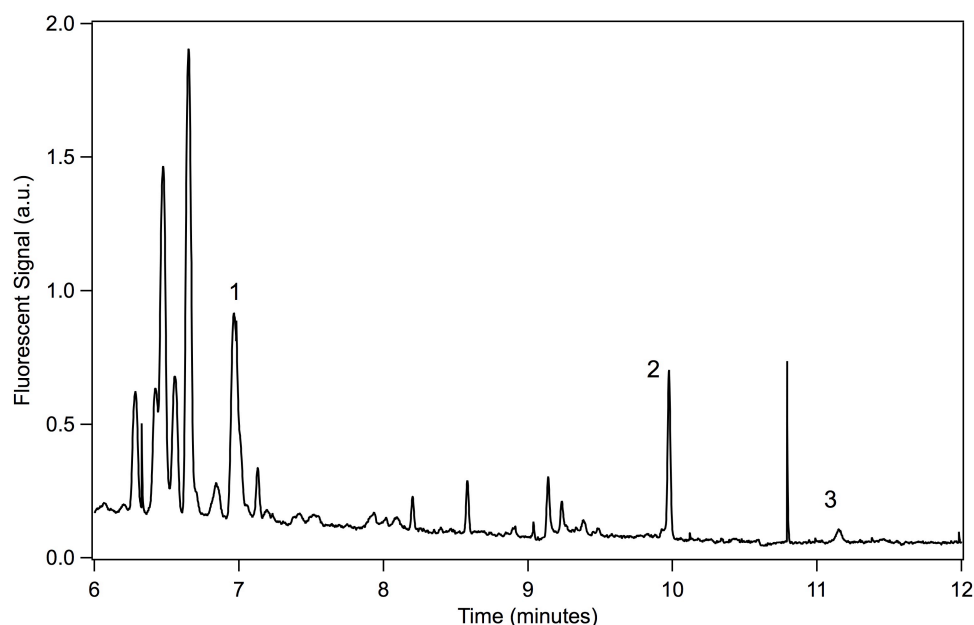


Figure 5.24 Electropherogram of dye extract from yellow thread from the Safavid fragment using methanol: distilled water (1:1, v/v) peaks were assigned as: lu-7-*O*-glu (peak 1), apigenin (peak 2) and luteolin (peak 3). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

Safavid Brocade: Red thread

The CE separation of the dye extract from the red thread is shown in **Figure 5.25**. In order to assign the peak eluted in run-a (peak 1), a fresh solution used in run-a was spiked with standard solutions of alizarin and carmine 0.8 and 1.2 $\mu\text{g mL}^{-1}$, respectively. The spiking method did not help to identify the peak in the red thread, as it was eluted between alizarin (peak 1) and carmine (peak 3).

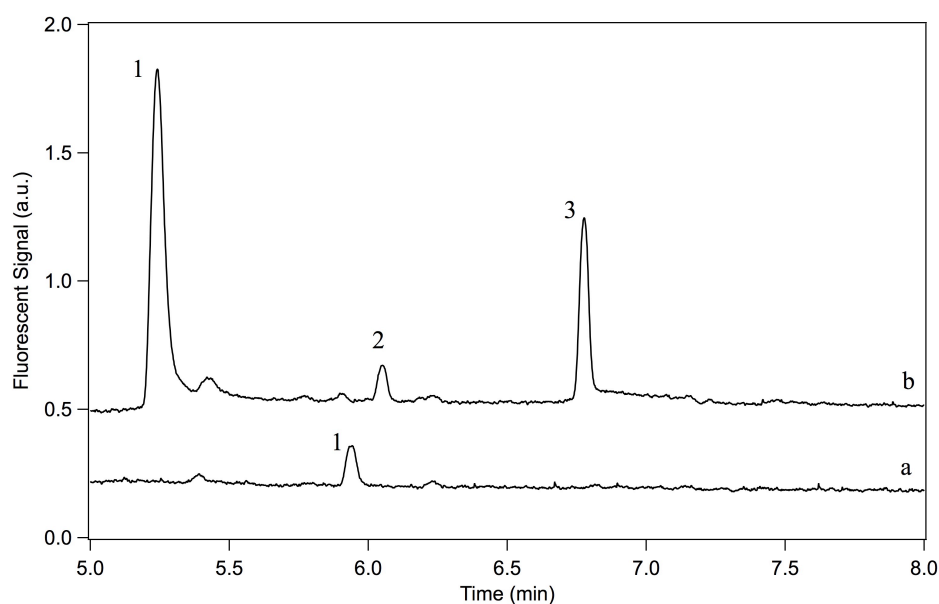


Figure 5.25 Electropherogram of **(a)** dye extract from red thread from the Safavid fragment using methanol: distilled water (1:1, v/v). **(b)** A fresh solution was spiked with standard solution of alizarin (peak 1) and carmine (peak 3). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV Reproduced with permission from Ref [45]. – Copyright: MDPI AG.

In this work the red dye could not be identified. However, it can be concluded that neither madder nor cochineal was used. By referring to the literature it can be inferred that it was lac dye [44]. The hypothesis suggesting that the red dye can be lac dye or kermesic acid since carminic

acid, kermesic acid were detected using HPLC-DAD in red Persian velvet textiles and red yarns of Persian carpet belonging to the 17th century A.D [18].

5.6.5 Antique Ukrainian tablecloth

An antique Ukrainian tablecloth sample was provided by the Winnipeg Art Gallery. One mg red thread was taken from the tablecloth and alizarin could be detected in the extract obtained from the sample. The results are given in **Figure 5.26**.

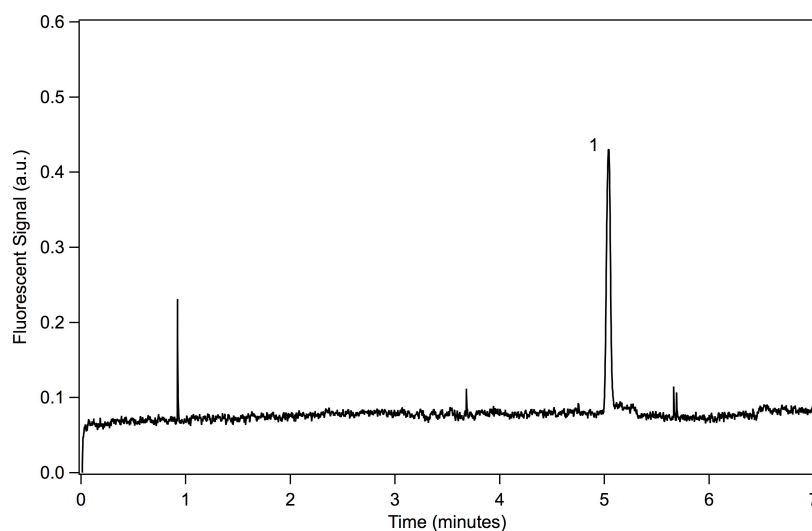


Figure 5.26 Electropherograms of dye extract from red thread belonging to the tablecloth. The peak was assigned as alizarin (peak 1). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV. Reproduced with permission from Ref [45]. – Copyright: MDPI AG

5.6.6 Wool dyed with walnut shell (*Juglans regia*)

The brown wool sample dyed with walnut was provided by Art University in Tehran, Iran.

One mg of sample was used for extraction of dyes following the procedure explained in previous section.

The results are given in **Figure 5.27**. Spiking the fresh sample with all flavonoid dyes helped to identify myricetin in the sample (peak 1).

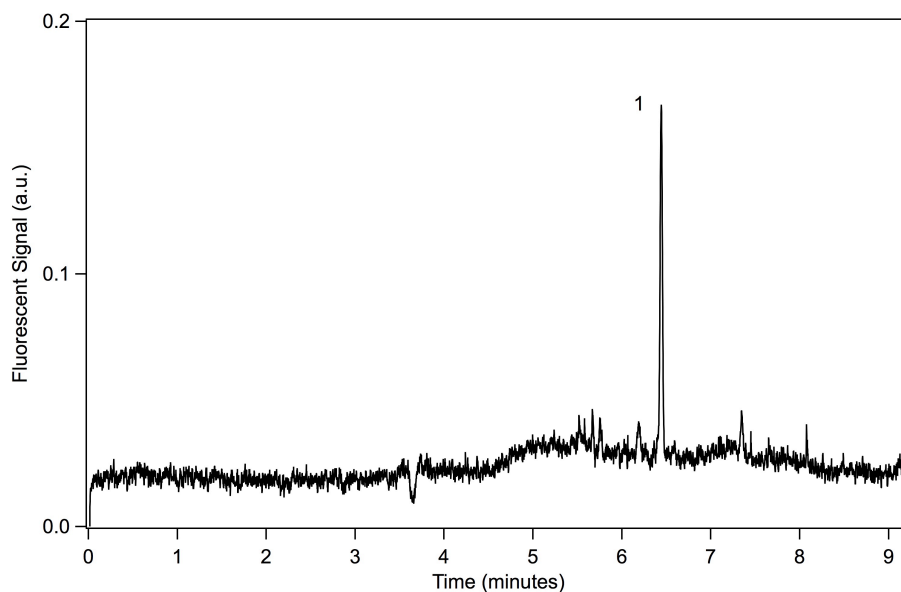


Figure 5.27 Electropherograms of dye extract from brown wool sample dyed with walnut. The peak was assigned as: myricetin (peak 1). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV. Reproduced with permission from Ref [45]. – Copyright: MDPI AG

5.6.7 Mordant studies

The effect of different mordants on extraction of dyes using methanol and water mixture from madder dyed wool samples was investigated. Therefore, the coarse hank wools was mordanted using following salts as described in section 5.1: alum: hydrated potassium aluminium sulfate ($\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$), tin: tin II chloride ($\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$), iron: iron II sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), copper: copper sulfate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) known as copper and chrome: potassium dichromate; $\text{K}_2\text{Cr}_2\text{O}_7$. Alizarin was extracted from madder dyed wool samples mordanted with different salts using methanol and water mixture following the procedure described in previous section. The visual observation

of the colour intensity of the extract solutions in DMSO indicated the following ranking: amount of dye extracted from the unmordanted wool > copper and chrome mordanted wools > iron mordanted wool > alum mordanted wool > tin mordanted sample. The CE- LIF was performed on the extracts and the results are given in **Figure 5.28**. The spiking showed that the peak number 1 belonged to alizarin. The results from the comparison of the runs are in agreement with the findings from visual observation.

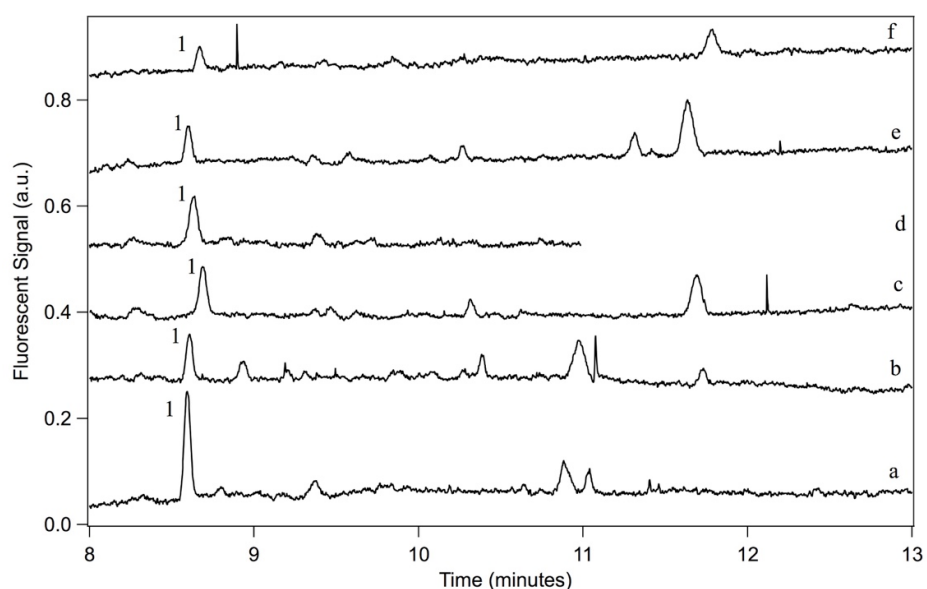


Figure 5.28 Electropherograms of dye extract from madder dyed wool samples: **(a)** unmordanted, **(b)** copper mordanted wool, **(c)** chrome mordanted wool, **(d)** iron mordanted wool, **(e)** alum mordanted wool, **(f)** tin mordanted sample. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

A comprehensive study was done by other researchers on the effect of different mordants on wash fastness of madder dyed wool samples. The wash fastness refers to the resistance of the dye to fading or running. The findings indicated that the wash fastness of the wool samples mordanted with chrome, tin, alum, and iron were in the same range (3-4) and higher than those of copper (2-3) [46].

5.6.8 Extracted dyes from papers

Several pieces of paper used for restoration of historical books dyed with different sources were provided by the Center of Document Restoration of Tehran University Library. The dyes were extracted from these samples using the mixture of methanol and water, following the procedure explained in previous section.

Paper dyed with henna

In order to identify the colourants in the light green colour paper dyed with henna, 2 μl of extract was dissolved in borate buffer and CE was performed, the results are given in **Figure 5.29**, run 'a'.

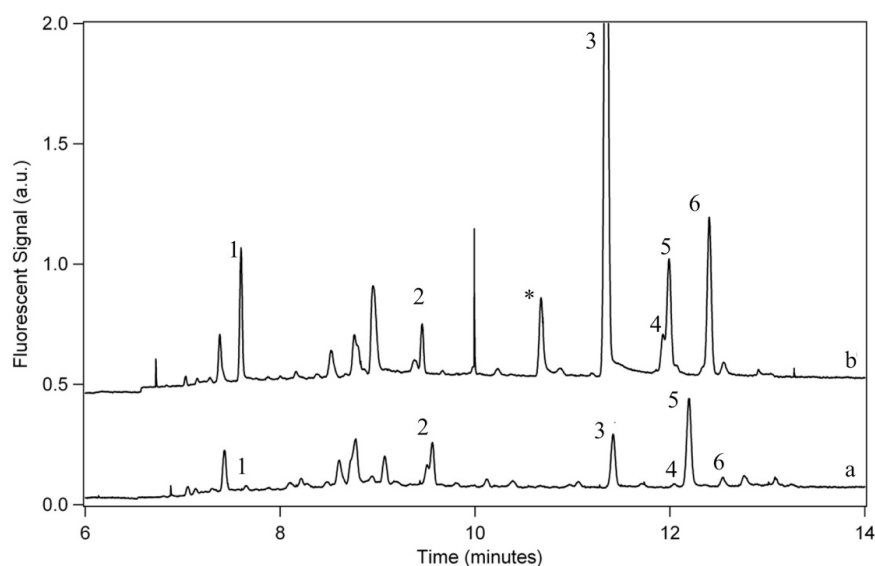


Figure 5.29 Electropherograms of (a) dye extract from paper dyed with henna using methanol: distilled water (1:1, v/v), (b) the sample from run a was spiked with standard solutions and the peaks were assigned as: lu-7-*O*-glu (peak 1), ap-7-*O*-glu (peak 2), Kaempferol (peak *), 0.2 $\mu\text{g ml}^{-1}$ apigenin (peak 3), 0.3 $\mu\text{g ml}^{-1}$ quercetin (peak 4), luteolin (peak 5) and morin (peak 6). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

To assign the peaks the same solution was spiked with the following standard solutions: $0.8 \mu\text{g ml}^{-1}$ lu-7-*O*-glu, ap-7-*O*-glu, $0.3 \mu\text{g ml}^{-1}$ Kaempferol, $0.2 \mu\text{g ml}^{-1}$ apigenin, $0.3 \mu\text{g ml}^{-1}$ quercetin, morin and luteolin (run b).

The comparison of run a and b confirmed that the colourants in the hennas were: lu-7-*O*-glu, ap-7-*O*-glu, apigenin, quercetin, luteolin and morin.

Paper dyed with Clove (*Syzygium aromaticum*)

The same method of extraction was applied to the light-yellow paper dyed with clove. Two μl of extract were used for the CE separation study. **Figure 5.30** run 'a' shows the separation of components in the extract of clove from the paper.

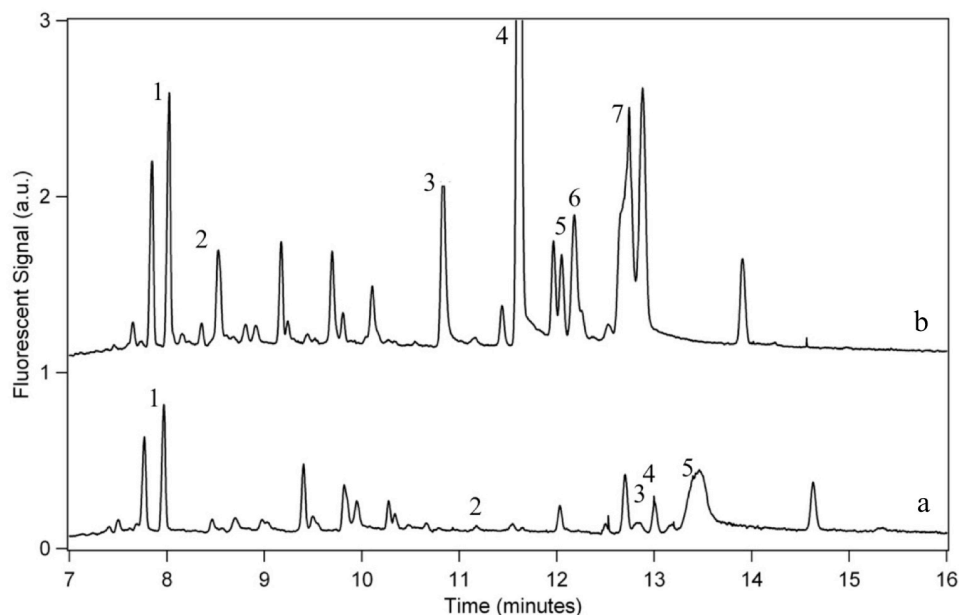


Figure 5.30 Electropherograms of (a) dye extract from paper dyed with clove using methanol: distilled water (1:1, v/v), (b) the sample from run a was spiked with standard solutions $1 \mu\text{g ml}^{-1}$ lu-7-*O*-glu and ap-7-*O*-glu, $0.5 \mu\text{g ml}^{-1}$ Kaempferol, $0.2 \mu\text{g ml}^{-1}$ apigenin, $0.5 \mu\text{g ml}^{-1}$ quercetin, $1 \mu\text{g ml}^{-1}$ luteolin, and $0.5 \mu\text{g ml}^{-1}$ morin. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

To identify the peaks, 0.5 ul of extract was used to spike with the standard solutions of flavones and flavonols: lu-7-*O*-glu, (peak 1), ap-7-*O*-glu (peak 2), kaempferol (peak 3), apigenin (peak 4), quercetin (peak 5), luteolin (peak 6) and morin (peak 7).

By comparison of run 'a' and 'b' it can be concluded that the main colourants in clove in run 'a' were: lu-7-*O*-glu (peak 1), kaempferol (peak 2), quercetin (peak3), luteolin (peak 4) and morin (peak 5).

In order to confirm the presence of luteolin, CE was performed on the another fresh solution of extract in borate buffer (ran a, **Figure 5.31**) and it was spiked with standard solutions of lu-7-*O*-glu, ap-7-*O*-glu, apigenin, and luteolin (run b). The comparison of the runs confirmed that peak 1 and 2 in run 'a' belonged to lu-7-*O*-glu and luteolin respectively.

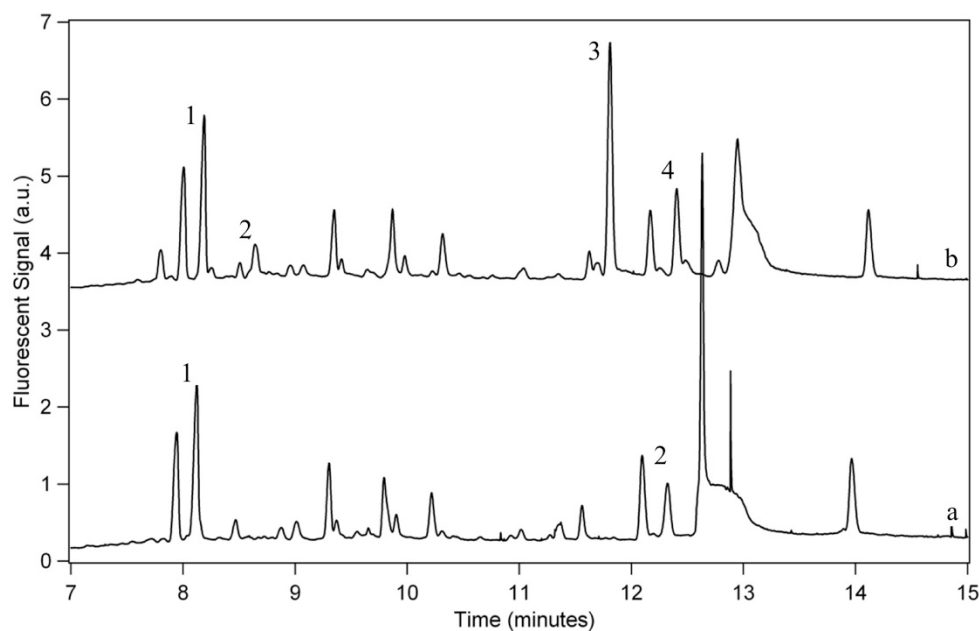


Figure 5.31 Electropherograms of (a) dye extract from paper dyed with clove using methanol: distilled water (1:1, v/v), (b) the sample from run a was spiked with standard solutions of $1 \mu\text{g ml}^{-1}$ lu-7-*O*-glu, ap-7-*O*-glu, $0.1 \mu\text{g ml}^{-1}$ apigenin, and $2 \mu\text{g ml}^{-1}$ luteolin. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

The phenolic colourants of clove were reported as flavonoids compounds including kaempferol and quercetin, were in agreement with the findings from this research [47].

Paper dyed with cochineal

The same method of extraction was applied on the light pink paper dyed with cochineal. Two μl of extract was used for the CE separation study. **Figure 5.32** run 'a' shows the separation of components in the extract. In order to identify the peaks, the same solution was spiked with the standard solution of carminic acid (run b). The comparison of runs 'a' and 'b' showed that the peak eluting at 8:40 minutes belonged to carminic acid.

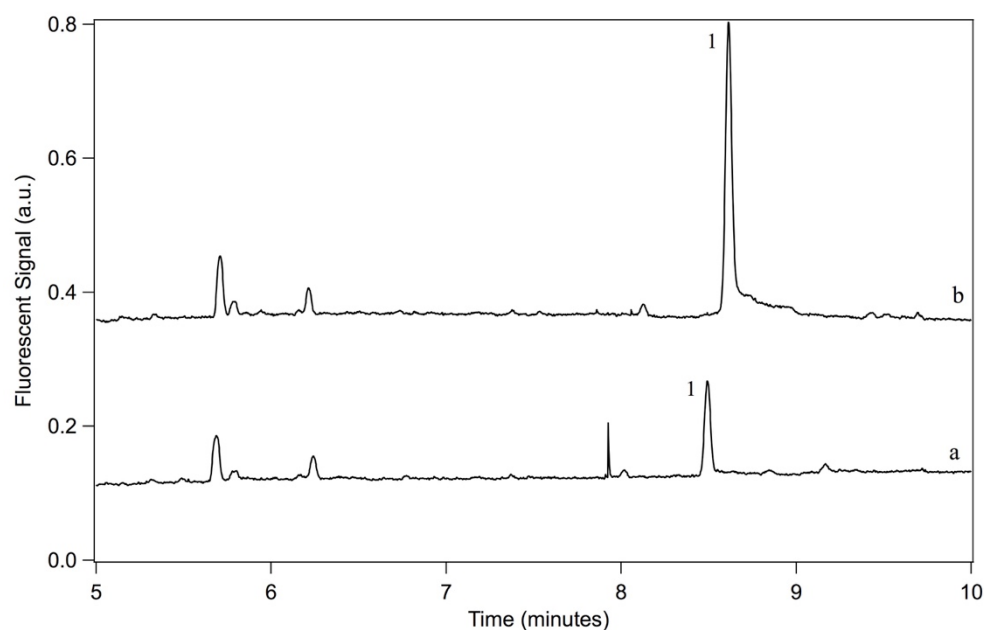


Figure 5.32 Electropherograms of (a) dye extract from paper dyed with cochineal using methanol: distilled water (1:1, v/v), (b) the sample from run a was spiked with standard solution of $3 \mu\text{g ml}^{-1}$ carmine. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

Paper dyed with madder

The dyes in 1mg light red paper dyed with madder were extracted using methanol and water. Two μl of extract was used for CE separation study. **Figure 5.33** run 'a' shows the separation of components in the extract. In order to identify the peaks, the same solution was spiked with the standard solution of alizarin (run b). The comparison of runs 'a' and 'b' showed that the peak 1 eluting at 6:40 minutes belonged to alizarin.

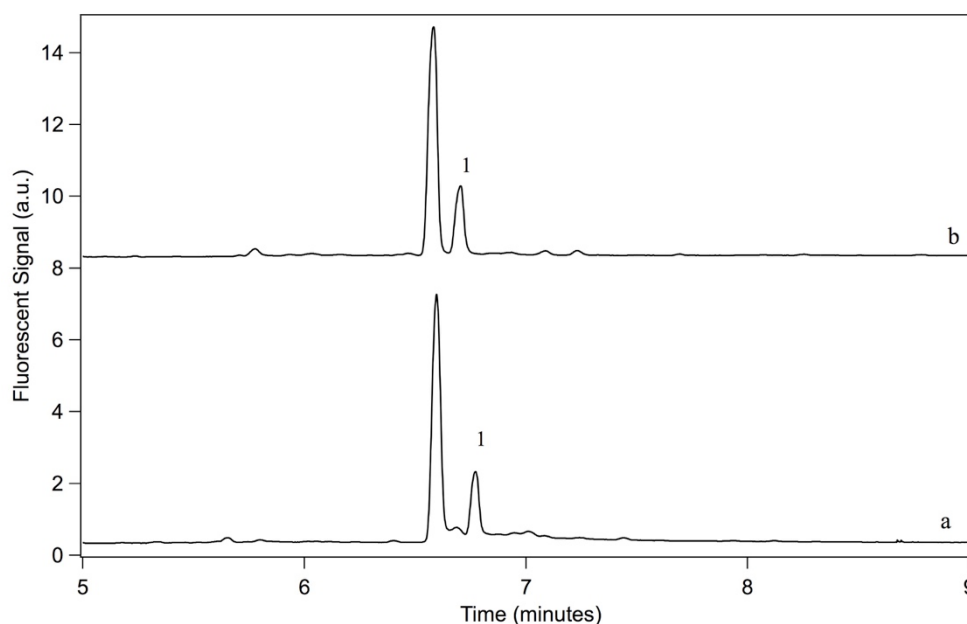


Figure 5.33 Electropherograms of dye extract **(a)** from paper dyed with madder using methanol: distilled water (1:1, v/v), **(b)** the sample from run a was spiked with standard solution of $5 \mu\text{g ml}^{-1}$ alizarin. Separation conditions 20 mM borate buffer at $\text{pH} = 8.5$; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

5.6.9 Dyes in the plants

In following the colourants in plants were extracted and analysed using the CE-LIF. The plants were: onion skin, henna, clove, weld, madder root, Persian berries, and walnut shell.

Onion skin

One gram of onion skin was left in 5ml DMSO for 10 minutes causing the solution to turn yellow. It was observed that 2ml of solvent were absorbed by the onion skin. The yellow extract was filtered by using a 45 μm filter and syringe. CE was performed on the solution of the 0.5 μl extract in the borate buffer (**Figure 5.34**, run a). A fresh solution made of 0.25 μl of filtered extract was spiked with kaempferol, myricetin, quercetin, morin, apigenin, and luteolin (run b). The comparison of the runs showed that peaks 1, 2 and 3 in run 'a' correspond to peaks 1, 4, and 5, were assigned as kaempferol, luteolin and quercetin, respectively.

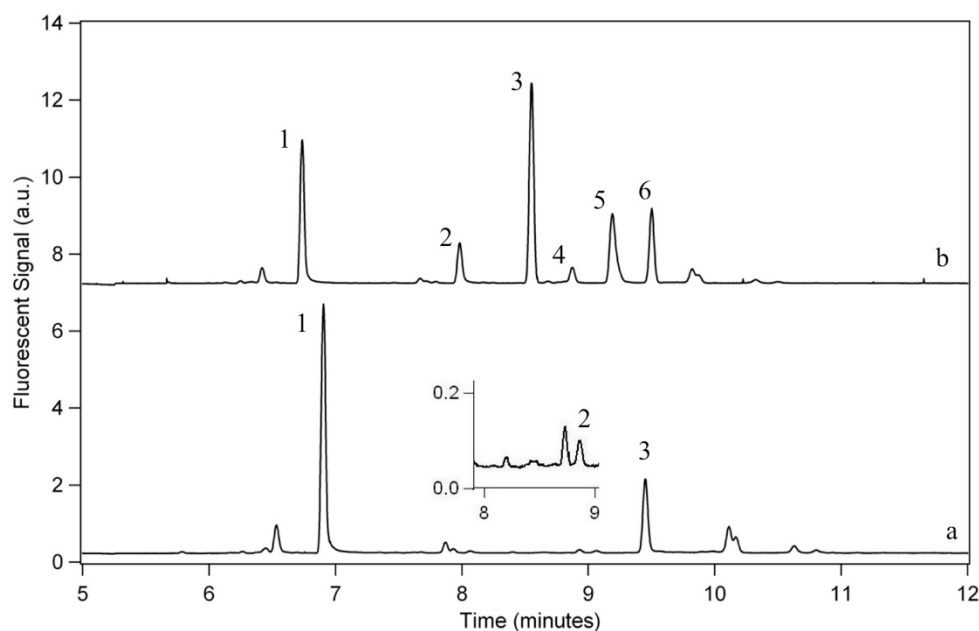


Figure 5.34 Electropherograms of dye extract from (a) onion skin using DMSO, (b) the sample from run a was spiked with standard solutions 2 $\mu\text{g ml}^{-1}$ kaempferol (peak 1), 1 $\mu\text{g ml}^{-1}$ myricetin (peak 2), 0.5 $\mu\text{g ml}^{-1}$ apigenin (peak 3), 1 $\mu\text{g ml}^{-1}$ luteolin (peak 4), 1 $\mu\text{g ml}^{-1}$ quercetin (peak 5), morin (peak 6) (run b). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

The results are in agreement with findings reported by other research group who extracted dyes using the harsh method, methanolic hydrochloric acid and milder methods including methanolic

formic acid and the mixture of EDTA, methanol and acetonitrile. Using the milder methods could identify quercetin-4'-o-glucoside as well as kaempferol and quercetin [12].

Henna (*lawsonia inermis* Inn)

One gram of henna was dissolved in 5 ml DMSO and left in a test tube for 1 day. Most plant powder precipitated and 4 ml of the supernatant was filtered using a syringe and filter (0.45 μ m). In return 0.5 μ l of this solution was dissolved in the borate buffer and CE was performed on the sample (**Figure 5.35**, run a). The same solution was spiked with standard solutions of kaempferol, apigenin, luteolin, quercetin and morin. The CE profile is given as run b. As opposed to the colourants were identified in the paper dyed with henna: apigenin, quercetin, luteolin and morin, this source of henna did not contain those colourants and none of the peaks were matched.

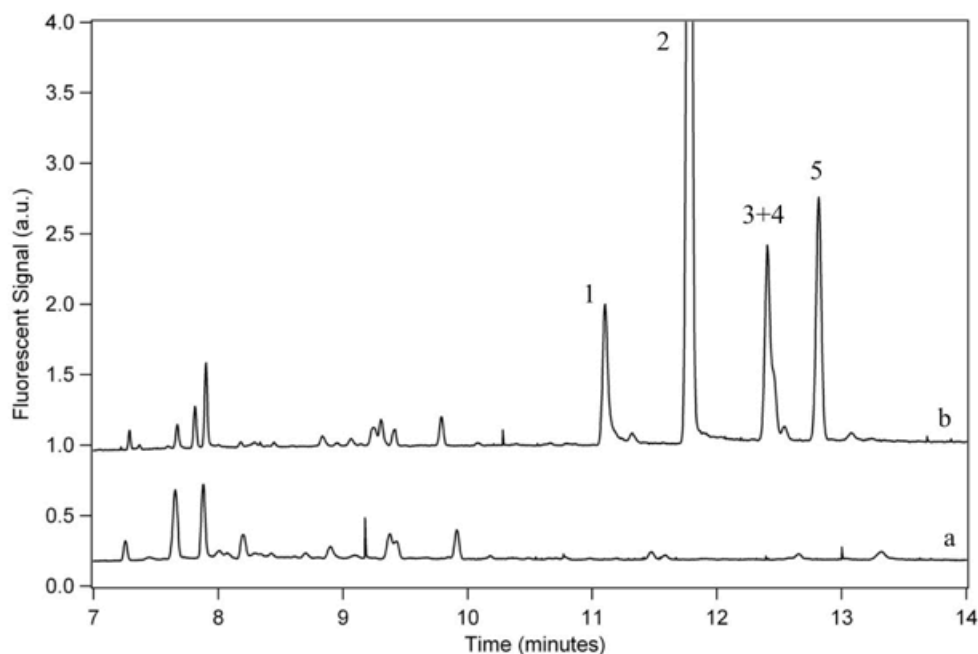


Figure 5.35 Electropherograms of (a) dye extract from henna using DMSO, (b) the sample from run a was spiked with standard solutions 0.5 $\mu\text{g ml}^{-1}$ Kaempferol (peak 1), 0.3 $\mu\text{g ml}^{-1}$ apigenin (peak 2), 0.5 $\mu\text{g ml}^{-1}$ luteolin and quercetin (peak 3 + 4), and morin (peak 5). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

In **Figure 5.35** since the dyes are in the region before kaempferol, there is a possibility that 1 or 2 of these peaks matched with lu-7-*O*-glu or ap-7-*O*-glu. CE was performed on the fresh solution (run a) after 24 hours **Figure 5.36**. A fresh solution was spiked with aforementioned dyes (run b). The comparison of 2 runs proved the presence of those dyes. Running CE on the extract from henna plant could confirm the presence of aglycone flavones, among other unidentified colourants. These findings are in agreement with the results from the study of chemical constituents of henna (*lawsonia inermis* Inn) [48]. The other research groups detected only luteolin and its 7-*O*-glucoside as well as other 3-*O*-glucoside of kaempferol, myricetin and quercetin [49].

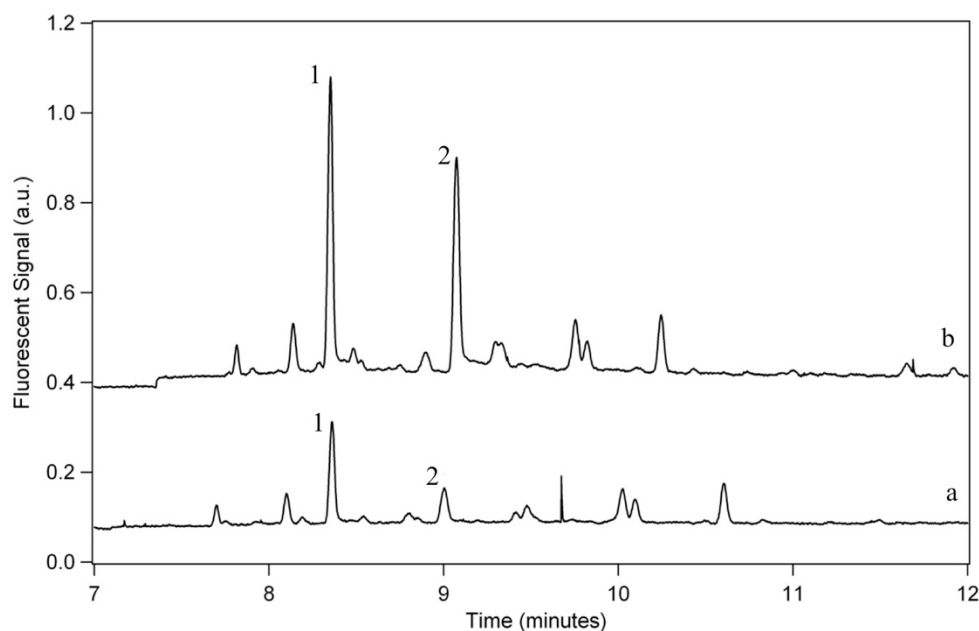


Figure 5.36 Electropherograms of (a) dye extract from henna using DMSO, (b) the sample from run a was spiked with standard solutions $1 \mu\text{g ml}^{-1}$ lu-7-*O*-glu and (peak 1) and ap-7-*O*-glu (peak 2). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

Weld (*Reseda luteola*)

The yellow dyes in weld plant were extracted using DMSO following the procedure used for henna plant. The fresh extract was spiked with all yellow standard flavonoid dyes and 3 peaks corresponding to lu-7-*O*-glu and (peak 1), ap-7-*O*-glu (peak 2), and luteolin (peak 3) could be assigned as shown in **Figure 5.37**.

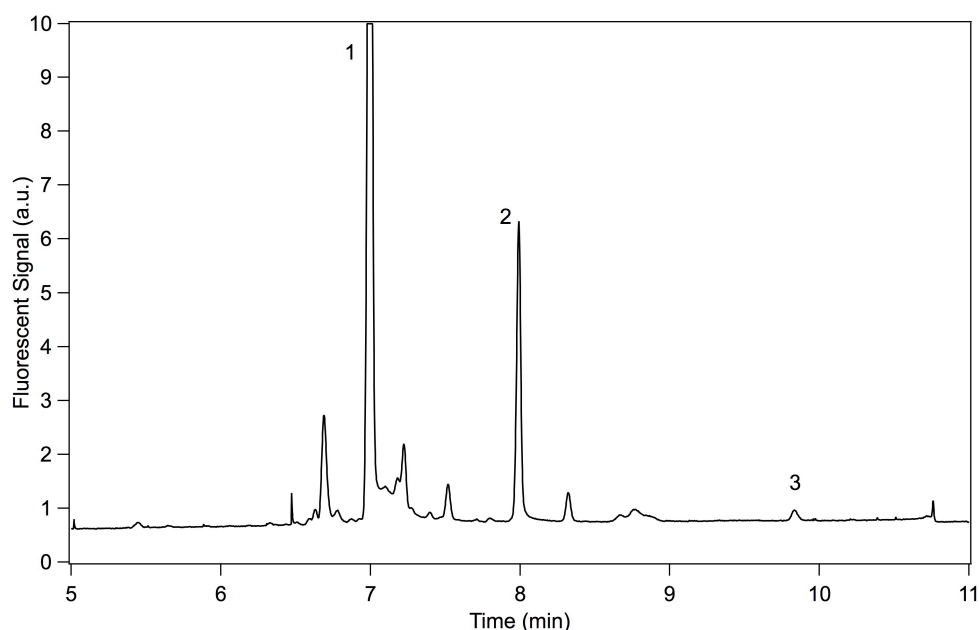


Figure 5.37 Electropherograms of dye extract from weld plant using DMSO. The peaks were assigned as: lu-7-*O*-glu and (peak 1), ap-7-*O*-glu (peak 2), and luteolin (peak 3). Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV.

Madder root (*Rubia tinctorum*)

For this experiment, a mild extraction was used in order to maximize the number of compounds detected. The madder root was purchased as a fine powder that had been previously dried. A 100 mg sample of powder was dissolved in 5 mL DMSO and heated gently (50°C) for 60 minutes with agitation. The mixture was allowed to cool for 1 hour with occasional stirring, prior to centrifuging

it for 5 minutes with a bench-top centrifuge. The clear solution was then diluted (5 μL in 0.5 mL) using 20 mM borate buffer (pH= 9.3) and the 0.5 mL samples were again centrifuged to avoid the injection of particulate matter into the capillary. **Figure 5.38** shows 2 electropherograms of alizarin extracted from madder (*Rubia tinctorum*) that was purchased in: (a) Tehran, Iran and (b) Mashhad, Iran. Although the 2 sources of powdered madder root were different in appearance with sample 1 being somewhat lighter in colour, their chemical properties with respect to alizarin and purpurin were quite similar.

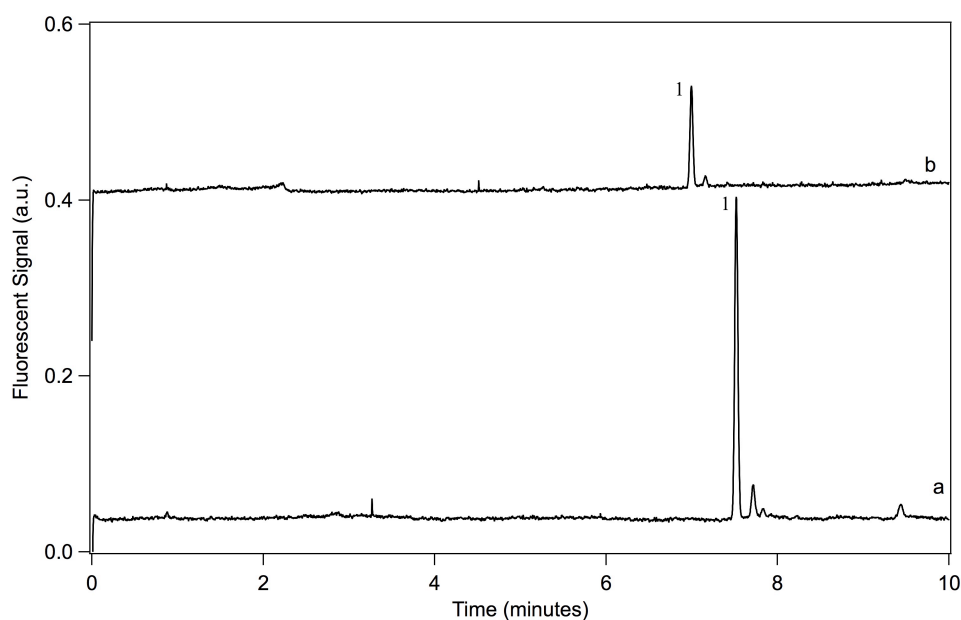


Figure 5.38 Electropherograms of alizarin extracted from powdered *Rubia tinctorum* (madder) from 2 sources **(a)** Tehran, Iran and **(b)** Mashhad, Iran. Separation conditions 20 mM borate buffer at pH = 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV. Adapted from Ref. [50] – Copyright: Taylor and Francis

Using an external calibration curve of peak heights, the concentration of alizarin in each madder root was determined to be 10.2 mg g⁻¹ and 10.7 mg g⁻¹ for source 1 and source 2 respectively. These concentrations of alizarin are similar to those reported by Zarkogianni *et al.* [51], who

reported the alizarin content in Madder root at 10.5 mg g^{-1} and Derksen *et al.* [52] who reported an alizarin concentration range between 6.1 and 11.8 mg g^{-1} . This is significantly higher than concentrations reported by others for concentrations of alizarin as 0.94 g kg^{-1} [53]. However, it has also been reported that the values for alizarin in madder root are quite variable [54]. Interestingly, purpurin and other anthroquinod dyes were not detected. This may indicate that other dyes such as purpurin were destroyed in the preparation of these powdered mixtures.

Persian berries (*Rhamnus catharic*)

The yellow dye in Persian berries were extracted using DMSO, and the dyes were separated using CE-LIF. The results are shown in **Figure 5.39**. The fresh extract was spiked with all yellow standard flavonoid dyes and the peaks eluting at 7:50 and 9:20 minutes were assigned as kaempferol and quercetin respectively.

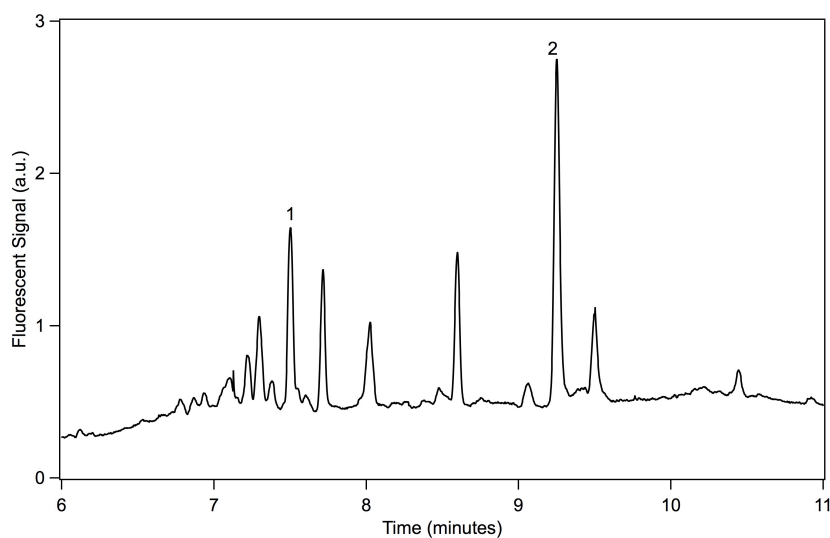


Figure 5.39 Electropherograms of dye extract from Persian berries using DMSO. The peaks were assigned as: kaempferol (peak 1) and quercetin (peak 2). Separation conditions 20 mM borate buffer at $\text{pH} = 8.5$; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV. Reproduced with permission from Ref [45]. – Copyright: MDPI AG

The results are in agreement with findings reported by other research groups, using LC-DAD to study dyes from different flavonoids dyes [16], [55].

5.7 Conclusion

The extraction of anthraquinones and flavonoids from wool samples were investigated using HCl, formic acid, methanol, DMSO, DMF and pyridine. The 6 extraction methods were compared on the basis of the CE-LIF results: number of the colourants extracted from the wool samples as well as the fluorescence intensity of the extracts. It was found that the application of the mixture of methanol and water resulted in extraction of both flavonoid glycones, aglycones and anthraquinone dyes. The application of methanol was preferred due to its lower boiling point, which allowed low temperature evaporation and drying the extract in the shortest time.

Based on the above, the methanol extraction method was selected and successfully applied on 1mg different samples: wool (“Salt Man” and carpet), silk (Safavid fragment and Chinese samples) and paper samples dyed with different natural plants. As mentioned before, the extraction efficiency can be affected by experimental conditions and it is an ongoing project to evaluate the application of different solvents at different conditions in term of using HPLC as the analysis technique. On Eu-ARTECH website [56] the information is shared. It is highly recommended that the same evaluation approaches used for HPLC studies are applied for CE-LIF [56]. Therefore, the research groups who are using CE-LIF can share their results and provide a very comprehensive data base.

As mentioned before, each dye has a unique chemical structure. Therefore, commercially available synthetic alizarin and alizarin obtained from plant material (madder) have the same chemical structure but the chromatograms obtained from these 2 sources would be quite different. The chromatogram obtained from a modern synthetic dye would be very simple containing only sharp peak belonging to the dye itself with only a few small peaks depending on the purity of the dye. However, it is expected that the chromatogram of dyes extracted from a plant, or historical object would have several peaks including the peak of the dye and the peaks of unwanted compounds extracted along with the dye.

The following is recommended for future studies:

- In this study, the yellow dye used in the Chinese silk fabric belonging to 1700 A.D. was not assigned due to the limitation of yellow standards in our data bank. Based on the sources of natural dyes reported for yellow colours in Chinese textiles, it is highly recommended to study the main colourants in Asian berberis: berberine, palmatin and Chinese yellow berries rutin (quercetin-3-o-glucoside).
- The red dye used for the wrapping the verso of the Safavid fragment belonging to 1700 A.D. was not identified. There is limited study on the different dyes used in Safavid textiles. It is recommended to study the CE-LIF properties of kermesic acid and lac dyes which are reported as 2 other sources of red dyes in 17th century.
- Moreover, the dye in blue threads belonging to Safavid brocade was not identified. In our lab 407 nm and 488 nm lasers light sources were available and none of these sources would be able to cause blue dyes to fluoresce. It is recommended to use different sources of lasers for identification of blue dyes using CE-LIF. However, organometallic approaches may

help to form complex compounds with the blue dyes, which fluoresce at 407 or 488 nm.

5.8 References

1. Samantaa, A.K. and P. Agarwal, *Application of natural dyes on textiles*. Indian Journal of Fibre & Textile Research, 2009. **34**: p. 384-399.
2. Schweppe, H., *Identification of dyes on old textiles*. Journal of the American Institute for Conservation, 1979. **19**(1): p. 14-23.
3. Karadağ, R. and E. Dölen, *Examination of historical textiles with dyestuff analyses by TLC and derivative spectrophotometry*. Turkish Journal of Chemistry, 1997. **21**(2): p. 126-133.
4. Wouters, J., *High performance liquid chromatography of anthraquinones: analysis of plant and insect extracts and dyed textiles*. Studies in Conservation, 1985. **30**(3): p. 119-128.
5. Wouters, J. and A. Verhecken, *The coccid insect dyes: HPLC and computerized diode-array analysis of dyed yarns*. Studies in Conservation, 1989. **34**(4): p. 189-200.
6. Halpine, S.M., *An improved dye and lake pigment analysis method for high-performance liquid chromatography and diode-array detector*. Studies in Conservation, 1996. **41**(2): p. 76-94.
7. Orska-Gawryś, J., et al., *Identification of natural dyes in archeological Coptic textiles by liquid chromatography with diode array detection*. Journal of Chromatography A, 2003. **989**(2): p. 239-248.
8. Surowiec, I., et al., *Identification of natural dyestuff in archeological coptic textiles by HPLC with fluorescence detection*. Analytical Letters, 2003. **36**(6): p. 1211-1229.
9. Trojanowicz, M., L. Wójcik, and K. Urbaniak-Walczak, *Identification of natural dyes in historical Coptic textiles by capillary electrophoresis with diode array detection*. Chemia analityczna, 2003. **48**(3): p. 607-620.
10. Surowiec, I., A. Quye, and M. Trojanowicz, *Liquid chromatography determination of natural dyes in extracts from historical Scottish textiles excavated from peat bogs*. J Chromatogr A, 2006. **1112**(1-2): p. 209-17.
11. Surowiec, I., B. Szostek, and M. Trojanowicz, *HPLC-MS of anthraquinoids, flavonoids, and their degradation products in analysis of natural dyes in archeological objects*. J Sep Sci, 2007. **30**(13): p. 2070-9.
12. Zhang, X. and R.A. Laursen, *Development of mild extraction methods for the analysis of natural dyes in textiles of historical interest using LC-diode array detector-MS*. Analytical Chemistry, 2005. **77**(7): p. 2022-2025.
13. Peggie, D.A., et al., *Towards the identification of characteristic minor components from textiles dyed with weld (*Reseda luteola* L.) and those dyed with Mexican cochineal (*Dactylopius coccus* Costa)*. Microchimica Acta, 2007. **162**(3-4): p. 371-380.
14. Sanyova, J., *Mild extraction of dyes by hydrofluoric acid in routine analysis of historical paint micro-samples*. Microchimica Acta, 2007. **162**(3-4): p. 361-370.

15. Valianou, L., I. Karapanagiotis, and Y. Chrysoulakis, *Comparison of extraction methods for the analysis of natural dyes in historical textiles by high-performance liquid chromatography*. Anal Bioanal Chem, 2009. **395**(7): p. 2175-89.
16. Petroviciu, I., F. Albu, and A. Medvedovici, *LC/MS and LC/MS/MS based protocol for identification of dyes in historic textiles*. Microchemical Journal, 2010. **95**(2): p. 247-254.
17. Liu, J., et al., *Identification of ancient textiles from Yingpan, Xinjiang, by multiple analytical techniques*. Journal of Archaeological Science, 2011. **38**(7): p. 1763-1770.
18. Serrano, A., et al., *Analysis of natural red dyes (cochineal) in textiles of historical importance using HPLC and multivariate data analysis*. Analytical and bioanalytical chemistry, 2011. **401**(2): p. 735-743.
19. Manhita, A., et al., *Extracting natural dyes from wool—an evaluation of extraction methods*. Analytical and bioanalytical chemistry, 2011. **400**(5): p. 1501-1514.
20. Taylor, G.W., *Detection and identification of dyes on Anglo-Scandinavian textiles*. Studies in Conservation, 1983. **28**(4): p. 153-160.
21. López-Montes, A., et al., *Simultaneous identification of natural dyes in the collection of drawings and maps from The Royal Chancellery Archives in Granada (Spain) by CE. Electrophoresis*, 2007. **28**(8): p. 1243-1251.
22. Maguregui, M.I., et al., *Micellar electrokinetic chromatography method for the determination of several natural red dyestuff and lake pigments used in art work*. J Chromatogr A, 2007. **1154**(1-2): p. 429-36.
23. Schweppe, H., *Identification of dyes in historic textile materials*, in *Historic textile and paper materials: Conservation and characterization*. 1986, American Chemical Society. p. 153-174.
24. Reichardt, C. and T. Welton, *Solvents and solvent effects in organic chemistry*. 2011: John Wiley & Sons.
25. Fardi, T., et al., *Artwork conservation materials and Hansen solubility parameters: A novel methodology towards critical solvent selection*. Journal of Cultural Heritage, 2014. **15**(6): p. 583-594.
26. Hansen, C.M., *Hansen solubility parameters: a user's handbook*. 2007: CRC press.
27. Hansen, C.M., *50 Years with solubility parameters—past and future*. Progress in Organic Coatings, 2004. **51**(1): p. 77-84.
28. Stefanis, E. and C. Panayiotou, *Prediction of Hansen Solubility Parameters with a New Group-Contribution Method*. International Journal of Thermophysics, 2008. **29**(2): p. 568-585.
29. Kharbade, B. and O. Agrawal, *Analysis of natural dyes in Indian historic textiles*. Studies in conservation, 1988: p. 1-8.
30. Clementi, C., et al., *A spectrometric and chromatographic approach to the study of ageing of madder (Rubia tinctorum L.) dyestuff on wool*. Analytica chimica acta, 2007. **596**(1): p. 46-54.
31. Rodríguez, E.S., Angela Arteaga Rodríguez, María Antonia García, and Rodríguez Carmen Cámara *Characterization of Natural and Synthetic Dyes Employed in the Manufacture of Chinese Garment Pieces by C-DAD and LC-DAD-QTOF*. e-conservation, 2011/09/. **21**, 38-55.

32. Yinon, J., M.E. Sigman, and M. Huang, *Forensic identification of dyes extracted from textile fibers by liquid chromatography mass spectrometry (LC-MS)*. Journal of Forensic Science, 2004. **49**(2): p. 1-12.
33. Haghi, G. and A. Hatami, *Simultaneous quantification of flavonoids and phenolic acids in plant materials by a newly developed isocratic high-performance liquid chromatography approach*. Journal of agricultural and food chemistry, 2010. **58**(20): p. 10812-10816.
34. Halpine, S.M., *Amino acid analysis of proteinaceous media from Cosimo Tura's 'The Annunciation with Saint Francis and Saint Louis of Toulouse'*. Studies in Conservation, 1992. **37**(1): p. 22-38.
35. Hadian, M., I. Good, and A. Pollard, *Textiles from Douzlakh Salt Mine at Chehr Abad, Iran: A Technical and Contextual Study of Late pre-Islamic Iranian Textiles*. The International Journal of Humanities, 2013. **19**(3): p. 152-173.
36. Gürses, A., et al., *Historical Development of Colorants*, in *Dyes and Pigments*. 2016, Springer. p. 1-12.
37. Vatandoust, A. and M.H. Dehkordi, "Saltman" a new archaeological discovery: scientific investigation and conservation. Journal of Biological Research Bollettino della Societa Italiana di Biologia Sperimentale, 2005. **1**(80): p. 236-242.
38. Öhrström, L.M., et al., *Radiological findings in an ancient Iranian salt mummy (Chehrābād ca. 410-350 BC)*. Skeletal radiology, 2015. **44**(6): p. 811-821.
39. Pollard, A., et al., *Below the salt: a preliminary study of the dating and biology of five salt-preserved bodies from Zanzan Province, Iran*. Iran, 2008: p. 135-150.
40. Vatandoust, A., *Saltman: Scientific Investigations Carried Out on Saltman Mummified Remains and Its Artifacts*. 1998.
41. Mouri, C., et al., *Analysis of dyes in textiles from the Chehrabad salt mine in Iran*. Heritage Science, 2014. **2**(1): p. 20.
42. Canby, S.R., *Shah 'Abbas: The Remaking of Iran*. 2009: British Museum Publications Limited.
43. Hardin, I.R. and F.J. Duffield, *Microanalysis of Persian textiles*. Iranian Studies, 1992. **25**(1-2): p. 43-59.
44. Shibayama, N., M. Wypyski, and E. Gagliardi-Mangilli, *Analysis of natural dyes and metal threads used in 16th -18th century Persian/Safavid and Indian/Mughal velvets by HPLC-PDA and SEM-EDS to investigate the system to differentiate velvets of these two cultures*. Heritage Science, 2015. **3**(1).
45. Ahmadi, S., D. Craig, and D. Goltz, *Micellar Electrokinetic Chromatography with Laser-Induced Fluorescence Detection for Separation of Red and Yellow Historical Dyes*. Chromatography, 2013. **1**(1): p. 9-23.
46. Feiz, M. and H. Norouzi, *Dyeing studies of wool fibers with madder (Rubia tinctorum) and effect of different mordants and mordanting procedures on color characteristics of dyed samples*. Fibers and Polymers, 2014. **15**(12): p. 2504.
47. Canameres, M., et al., *Surface-enhanced Raman scattering study of the anthraquinone red pigment carminic acid*. Vibrational spectroscopy, 2006. **40**(2): p. 161-167.
48. Chaudhary, G., S. Goyal, and P. Poonia, *Lawsonia inermis Linnaeus: a phytopharmacological review*. Int J Pharm Sci Drug Res, 2010. **2**(2): p. 91-8.

49. Dhaouadi, K., et al., *Commercial Lawsonia inermis L. dried leaves and processed powder: Phytochemical composition, antioxidant, antibacterial, and allelopathic activities*. Industrial Crops and Products, 2015. **77**: p. 544-552.
50. Goltz, D.M., et al., *Separation of historical dyes using capillary electrophoresis with laser-induced fluorescence detection*. Journal of Liquid Chromatography & Related Technologies, 2012. **35**(14): p. 2054-2065.
51. Zarkogianni, M., Z.-E. Papiaka, and E. Tsatsaroni, *Identification and quantitative determination of madder by high performance liquid chromatography: application to historical textiles*. Journal of Liquid Chromatography & Related Technologies®, 2009. **32**(16): p. 2334-2345.
52. Derksen, G.C., et al., *Two validated HPLC methods for the quantification of alizarin and other anthraquinones in Rubia tinctorum cultivars*. Phytochemical Analysis, 2004. **15**(6): p. 397-406.
53. Lodhi, A., A. Sant'Ana, and B. Charlwood, *Quantitative analysis of alizarin in tissue cultures of Rubia species by high performance liquid chromatography*. Phytochemical Analysis, 1994. **5**(5): p. 261-265.
54. Boldizsár, I., et al., *Identification and quantification of the constituents of madder root by gas chromatography and high-performance liquid chromatography*. Journal of Chromatography A, 2006. **1133**(1): p. 259-274.
55. Mouri, C., et al., *Characterization of flavonols in plants used for textile dyeing and the significance of flavonol conjugates*. Dyes and Pigments, 2014. **100**: p. 135-141.
56. <http://www.eu-artech.org/>.

Chapter 6

Conclusion

6.1 Conclusion

The objective of this doctoral research was to develop an innovative and novel methodology for the separation and identification of natural dyes in historical objects (textiles) using CE equipped with laser induced fluorescence (CE-LIF); as well as to validate existing extraction methods with special focus on Hansen solubility parameters.

One of the most important principles in art conservation is to maintain the integrity of objects. Therefore, conservation scientists strive to achieve this goal by applying an analytical technique which needs the smallest amount of sample to minimize the damage to valuable objects. The identification of minute amounts of natural dyes in historical textiles could be achieved by means of CE-LIF due to its high sensitivity using fluorescence technique and the following goals were achieved:

Capillary electrophoresis equipped with laser induced fluorescence separations can be affected by buffer concentration, buffer pH, application of micelles; modifiers, and applied voltage. A separation procedure for the mixture of historically important dyes was developed by manipulating the separation factors. The optimum separation condition was found to be: 20 mM borate buffer at pH= 8.5; 20 mM SDS with 5% acetonitrile and an applied potential of 22 kV. Reproducible electropherograms of standard solutions of pure dyes under alkaline conditions (at pH= 8.5) were achieved. Purpurin and flavonols at pH>8.5 did not show reproducible peaks. It was suspected that some degradation was occurring, therefore, their UV-Vis absorption properties under alkaline conditions were evaluated to understand if any changes in chemical structure occurred. The poor peak reproducibility of CE separations for these dyes at pH>8.5 was attributed to their photochemical

properties. The rate constants for photochemical fading of flavonols and purpurin at pH= 7.0, and 9.2, in light and dark conditions was measured. The kinetics data showed that the rate of decomposition of these dyes increased by exposing to intense light and higher pH value. The 1st order rate constant indicated that a photochemical reaction was the cause of decomposition of these dyes. The photodegraded products of purpurin were identified as: phthalic acid and salicylic acid by thin layer chromatography, NMR, and GC-MS. To minimize the effect of light on degradation of dyes, it was concluded that for CE separation the samples should be prepared freshly and the stock solutions should be stored in a dark condition.

Another objective of this research was to optimize a procedure for extraction of dyes from a minute sample. The existing extraction methods (different solvents and extraction conditions: time, temperature, agitation) were validated on laboratory wool samples dyed using weld and madder, and the extraction efficiency was evaluated by Hansen solubility parameters as well as running CE on extracted samples. The results showed that the application of the mixture of methanol and distilled water, at 92°C for 30 minutes, with ultrasonication appeared to be promising for the extraction of all dyes under this study.

The optimum separation procedure and methanol extraction were validated on genuine minute samples $\leq 1\text{mg}$ including: wool threads from a piece of hand woven carpet (100-year-old), silk threads from a Chinese robe belonging to 1700 AD, wool threads from clothing of the “Salt Man” (600 BC), piece of cloth from Safavid time (1700 AD), and a red thread of a table cloth belonging to the 20th century. The optimum separation conditions were used successfully for identification of dyes in plants: onion skin, henna, clove, madder, weld, Persian berries; as well as for evaluation of the role of different mordants (Al^{3+} , Cu^{2+} , Cr^{3+} , Fe^{3+} and Sn^{2+}) on wash fastness of madder dyed

wool samples. Generally speaking, CE-LIF was useful for the separation and identification of small quantities of natural dyes extracted from less than 1 mg samples including wool, silk, and paper. In this research, it was showed that methanol could be used to extract dyes from less than 0.5 mg of genuine textiles. Methanol extraction was considered as a minimally destructive method. Furthermore, the extracted dyes from the minute samples could also be extracted for further studies. This opportunity was possible because of the high sensitivity and low detection limit of CE-LIF (pg). As opposed to HPLC and GC that the required larger amounts of sample (0.5 to 1 mg) with the detection limits in the range of ng. The results from GC-MS are comparable with CE while the application of CE has an advantage over GC, which does not need the derivatization. Vibrational techniques (IR, RAMAN) had disadvantages of spectral interferences from the substrate itself. Furthermore, CE had much better detection limits than spectroscopic approaches including UV-Vis spectrophotometry.

6.2 Future studies and outlook

The following subjects are recommended for further studies:

It was observed that the dyes with similar pK_a and chemical structure showed co-elution or displacement in elution order by using a pH close to their pK_a values. The changes in elution was due to the influence of pH on mobility and retention factor of the dyes, whereby increasing pH, the retention factor (k') and the mobility of more hydrophobic compounds declined [1]. In order to study the changes in elution order, retention factor (k') should be calculated using Eq. 6.1 [1] :

$$k' = \frac{(t_R - t_0)}{t_0 \left(1 - t_R/t_{mc}\right)} \quad \text{Eq. 6.1}$$

where: t_R = dye migration time (minute), t_0 = buffer solution migration time (minute), and t_{mc} = micelle migration time (minute)

In order to measure the migration time of buffer solution (t_0) methanol is used, since its k' between micelle and buffer is very small and methanol can be detected by UV detector. Sudan III or IV are used to trace SDS and measure t_{mc} . When CE-LIF is employed t_0 and t_{mc} cannot be measured since neither methanol nor Sudan III or IV fluoresce [2]. Therefore, the change of the elution order can be systematically studied by CE equipped with other schematic detectors e.g. DAD is recommended.

In Chapter 2, the effect of 3 modifiers: methanol, acetonitrile and isopropanol were studied on the fluorescence properties of alizarin, purpurin, carmine and morin. The impact of these solvents on the fluorescence of the dyes could be explained in regard to intra and inter molecular hydrogen bonding interactions. It was observed that fluorescence intensity of purpurin in the presence of acetonitrile was greater than alizarin. Alizarin is more stable in the ground state and it forms intramolecular hydrogen bonds, while in the excited state a proton transfer (ESPT) happens, and hydrogen transfers from the phenolic OH group to the carbonyl group, which changes the molecular geometry of alizarin. In terms of purpurin, the intramolecular hydrogen bond remained in excited state. It is recommended to study the effect of these modifiers as well as SDS on fluorescence properties of all dyes.

The metal cations such as: Mg^{2+} , Zn^{2+} , Ca^{2+} , Be^{2+} , Al^{3+} , In^{3+} , Ga^{3+} , Zr^{4+} , have been used to achieve lower detection limits in spectrophotometry and fluorometry studies of dyes [3-9]. It is recommended to investigate if the formation of metal complexes:

- will improve the fluorescence properties of some dyes such as indigo, curcumin, henna while the 407 nm laser source is used (or other available laser sources). Also, if forming complex will stabilize some dyes such as purpurin, myricetin, and quercetin in alkaline conditions ($\text{pH} > 9.2$). Hollman *et al.* showed that the fluorescence intensity of flavonols specially quercetin increased by forming Al^{3+} complex and the flavonols could be separated and identified using HPLC equipped with fluorescence detection [6]. The effect of Be^{2+} on the improving the fluorescence intensity of some flavonols have also been reported [10]
- will have a promising impact on mobility of dyes and enhance their separation efficiency. Široká *et al.* showed that the selectivity and sensitivity of CE separation could be improved by forming complexes between organic ligands such as flavonoids and metal ions [11].

The role of different mordants (alum, copper, chrome, iron and tin) was studied on the fastness of the madder dyed wool samples. The same study is recommended for wool, and silk samples dyed with other natural dyes such as: weld, clove, walnut shells, and carmine. This study would show the effect of different mordants on the fastness of silk and wool samples.

In this study the concentration of alizarin in 2 different madder sources were calculated. For future studies the concentrations of colouring matters in other plant sources: weld, clove, henna and onion skin would be calculated.

In this study by means of proton NMR, thin layer chromatography and gas chromatography, the degraded products in purpurin were identified. It is recommended to use the same techniques as well as CE equipped with MS to identify degradation products in flavonols [12], [13].

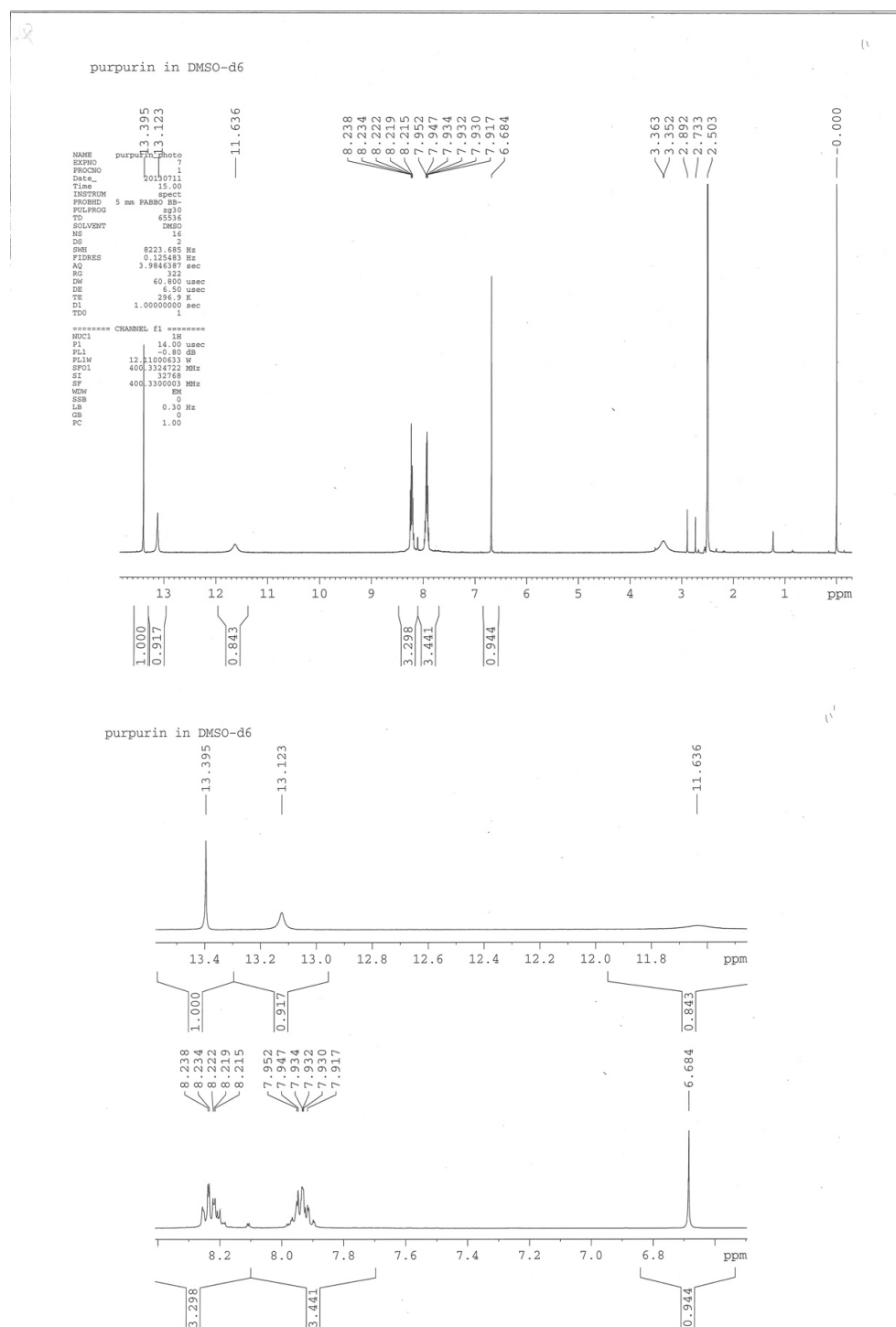
In case studies, the yellow dye used in clothing of “Salt Man” and in Chinese robe could not be identified due to the limited available data. Regarding the yellow wool samples from clothing of “Salt Man”, different centers have reported different colourants. It is recommended to extract dyes from tamarix plant, the local plant grows near the salt mine which is consisted of ellagic acid, quercetin glucuronide, quercetin 3-o-sulfate, kaempferol 3-o-sulfate, isorhamnetin or tamarixetin 3-o-sulfate, quercetin (3',4 or 7)-o-sulfate. Therefore, the study of the separation behavior of standard solutions of these dyes with CE-LIF, would be a proof for the findings by Mouri *et al.* [14]. According to published literature in China, in 1700 AD the textiles were dyed by Asian berberis and Chinese yellow berries to achieve yellow colour. It is recommended to study separation and identification of berberine, palmatin the main colourants in Asian berberis as well as rutin (quercetin-3-o-glucoside) the main colourant in Chinese yellow berries [15].

6.3 References

1. Khaledi, M.G., S.C. Smith, and J.K. Strasters, *Micellar electrokinetic capillary chromatography of acidic solutes: migration behavior and optimization strategies*. Analytical chemistry, 1991. **63**(17): p. 1820-1830.
2. Altria, K.D., *Capillary electrophoresis guidebook: principles, operation, and applications*. Vol. 52. 1996: Springer Science & Business Media.
3. Surowiec, I., W. Nowik, and M. Trojanowicz, *Post-column deprotonation and complexation in HPLC as a tool for identification and structure elucidation of compounds from natural dyes of historical importance*. Microchimica Acta, 2008. **162**(3-4): p. 393-404.

4. White, C.E., D.E. Hoffman, and J.S. Magee, *Fluorescence emission spectra, fluorescence excitation spectra, and absorption spectra of some metal chelates*. Spectrochimica Acta, 1957. **9**(2): p. 105-112.
5. White, C., M. Ho, and E. Weimer, *Fluorescence spectra of metal chelates*. Spectrochimica Acta, 1960. **16**(1-2): p. 236-237.
6. Hollman, P.C., J.M. van Trijp, and M.N. Buysman, *Fluorescence detection of flavonols in HPLC by postcolumn chelation with aluminum*. Analytical Chemistry, 1996. **68**(19): p. 3511-3515.
7. Katyal, M., *Flavones as analytical reagents—a review*. Talanta, 1968. **15**(1): p. 95-106.
8. Katyal, M. and S. Prakash, *Analytical reactions of hydroxyflavones*. Talanta, 1977. **24**(6): p. 367-375.
9. Sawada, T., T. Shibamoto, and H. Kamada, *Fluorescence Lifetime Measurements of Morin–Metal Ion Complexes*. Bulletin of the Chemical Society of Japan, 1978. **51**(6): p. 1736-1738.
10. Hayashi, T., et al., *Fluorometric Study on the Metal Chelates of Flavone Derivatives. I. Correlation between Fluorescence Intensity and Structure of Beryllium Chelates*. Chemical and Pharmaceutical Bulletin, 1970. **18**(6): p. 1112-1117.
11. Široká, J., P. Jáč, and M. Polášek, *Use of inorganic, complex-forming ions for selectivity enhancement in capillary electrophoretic separation of organic compounds*. TrAC Trends in Analytical Chemistry, 2011. **30**(1): p. 142-152.
12. Cuyckens, F. and M. Claeys, *Mass spectrometry in the structural analysis of flavonoids*. Journal of Mass spectrometry, 2004. **39**(1): p. 1-15.
13. Colombini, M.P., et al., *Colour fading in textiles: A model study on the decomposition of natural dyes*. Microchemical Journal, 2007. **85**(1): p. 174-182.
14. Mouri, C., et al., *Analysis of dyes in textiles from the Chehrabad salt mine in Iran*. Heritage Science, 2014. **2**(1): p. 20.
15. Rodríguez, E.S., Angela Arteaga Rodríguez, María Antonia García, and Rodríguez Carmen Cámara *Characterization of Natural and Synthetic Dyes Employed in the Manufacture of Chinese Garment Pieces by C-DAD and LC-DAD-QTOF*. e-conservation, 2011/09/. **21**, 38-55.

Appendix A: Supplementary information for Chapter 4

Figure A.4. 1 The ^1H NMR of purpurin in deuterated DMSO

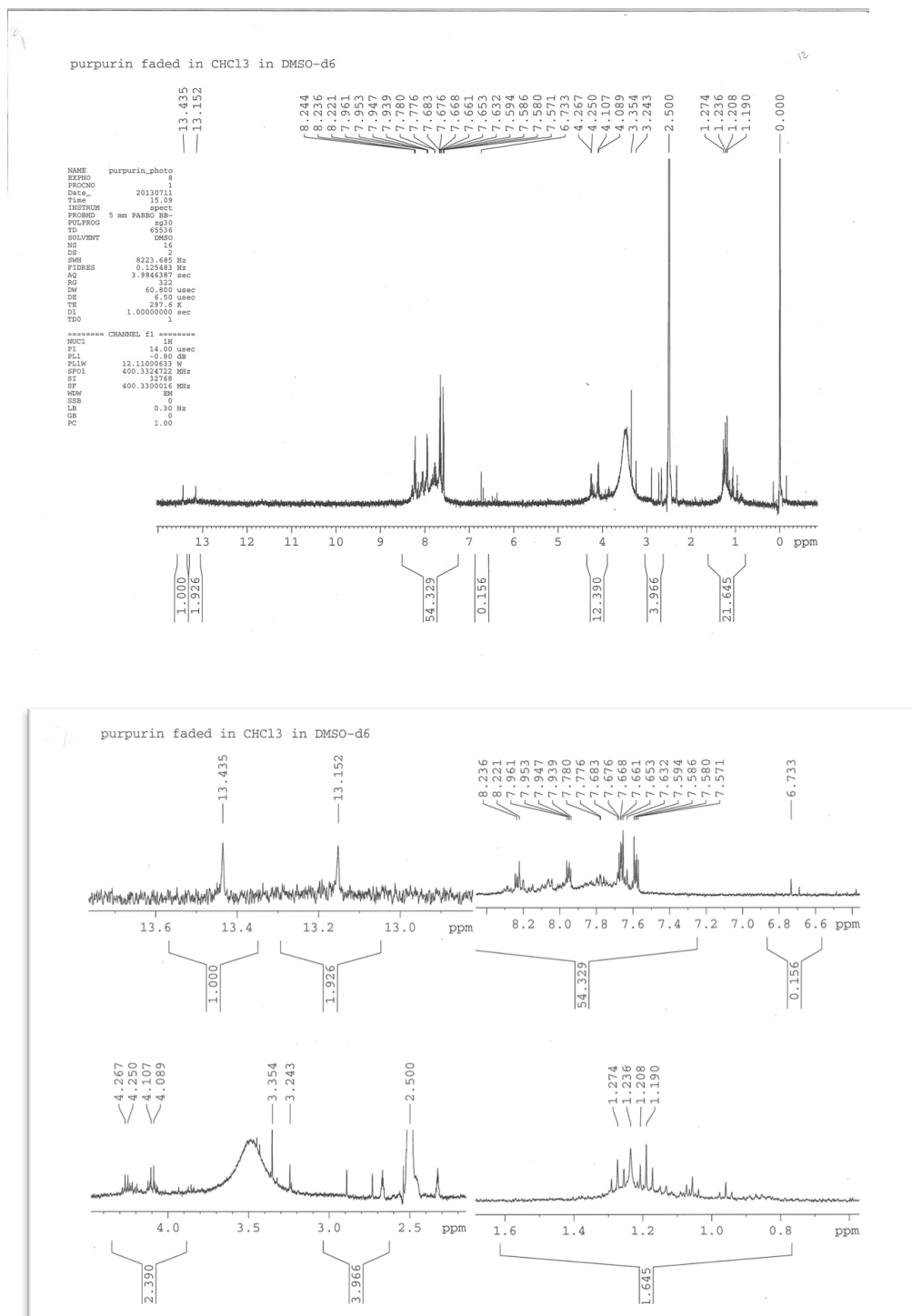


Figure A.4.2 The ¹H NMR of faded purpurin in deuterated DMSO

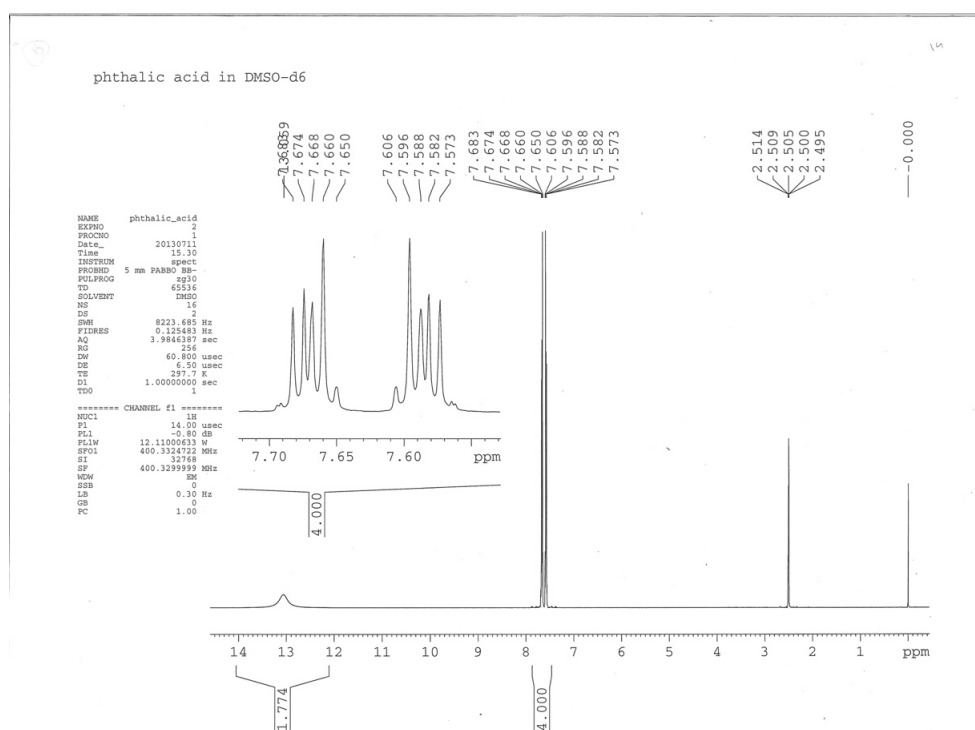


Figure A.6.3 The ^1H NMR of phthalic acid in deuterated DMSO

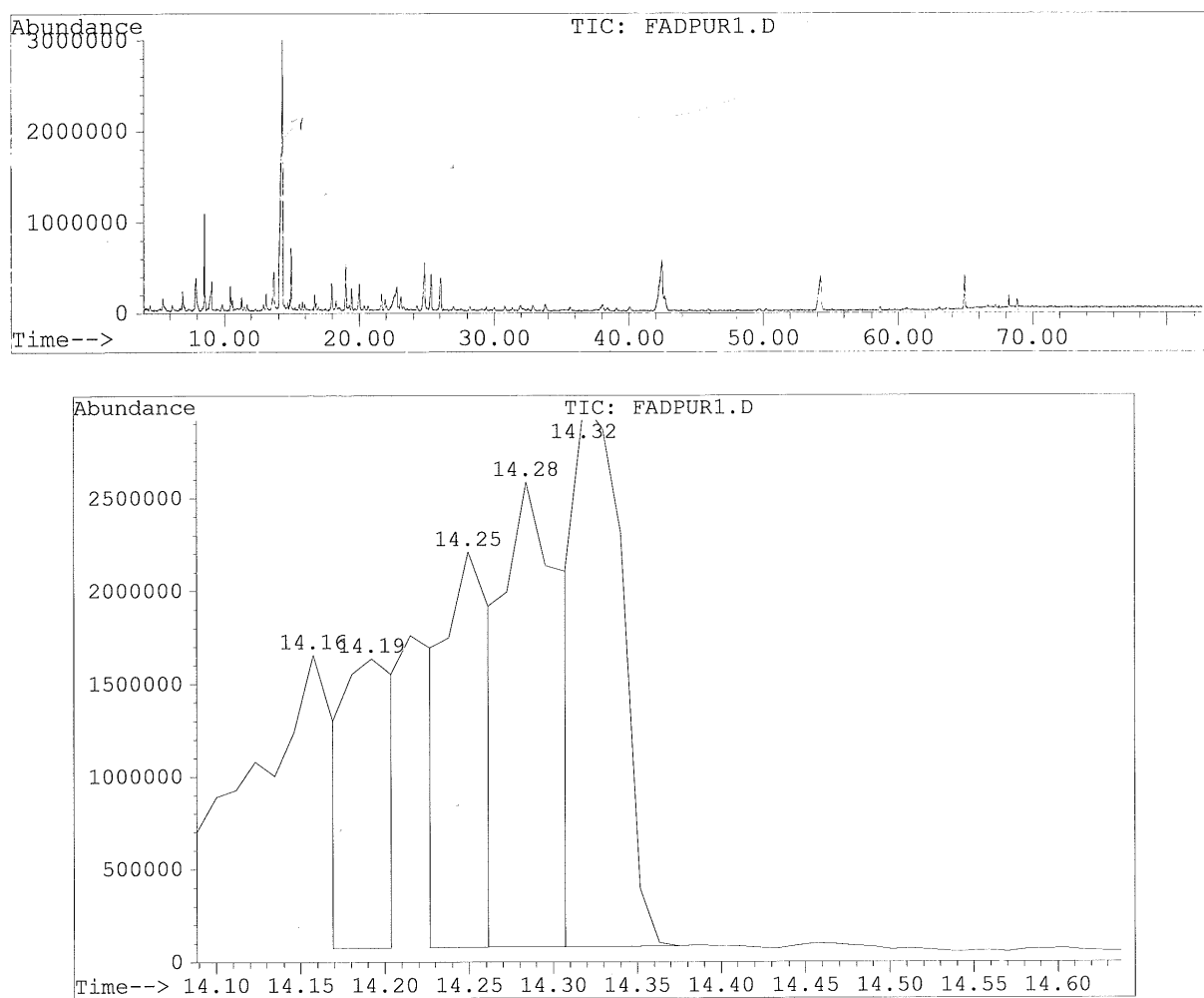


Figure A.6.4. a The gas chromatogram of faded purpurin

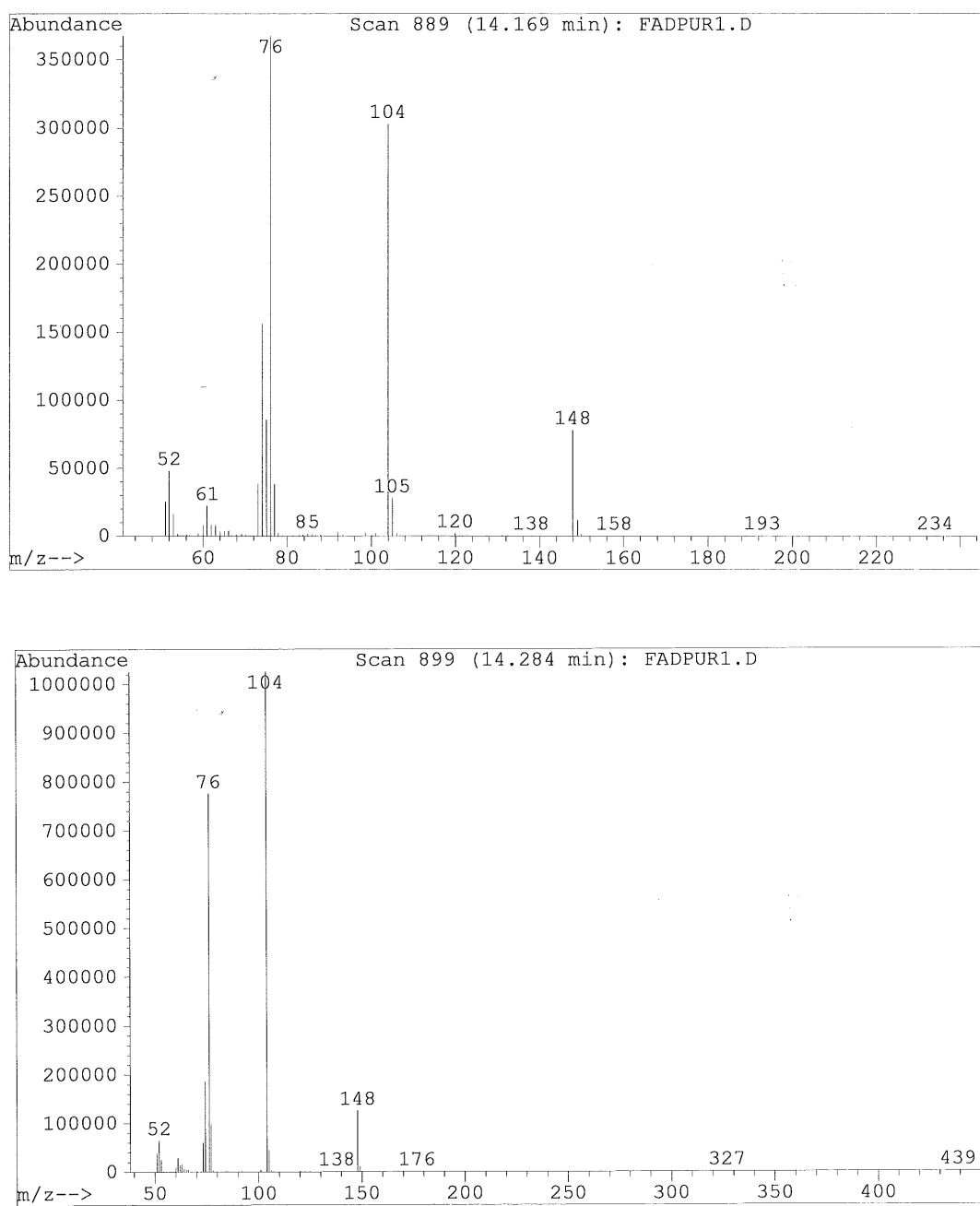


Figure 64.4. b The mass spectrum of peak at 14.169 and 14.284 minutes

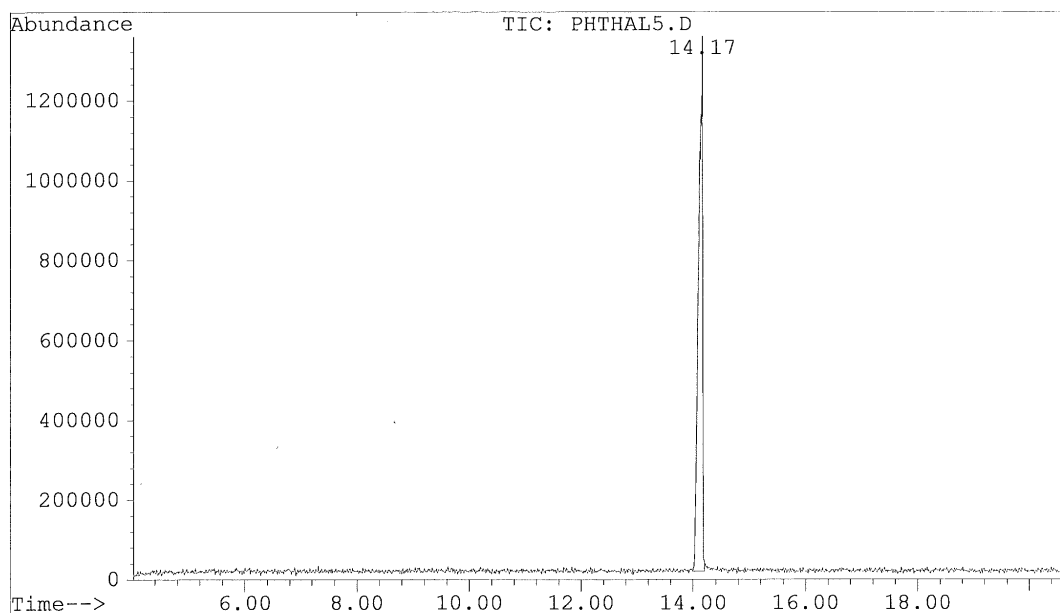


Figure 6.4.5. a The gas chromatogram of phthalic acid

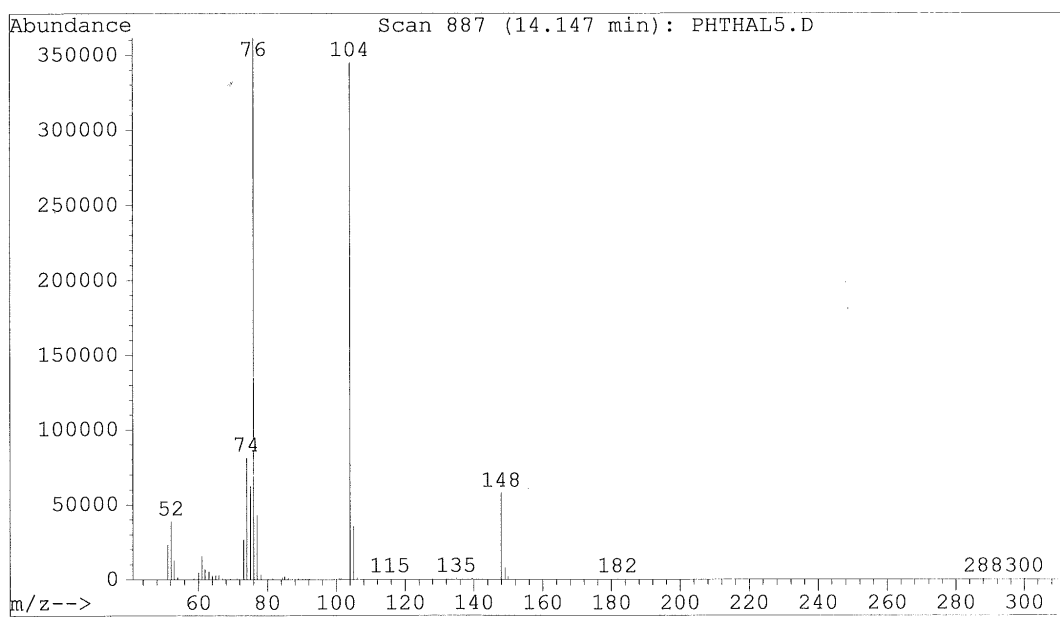


Figure A.6.5. b The mass spectrum of peak at 14.147 minute

Table A.4.1 Absorption characteristics of dyes in 8 µgml⁻¹ borate buffer, at pH values of 7.0, 9.2 and 11.0 in minute zero and in minute 60.

Dye	MW (g/mol)	PPM	M (mol/lit)	pH	Minute 0						Minute 60					
					λ_{max} (nm)	Abs	molar absorptivity (1 mol ⁻¹ cm ⁻¹)	λ_{max} (nm)	Abs	molar absorptivity (1 mol ⁻¹ cm ⁻¹)	λ_{max} (nm)	Abs	molar absorptivity (1 mol ⁻¹ cm ⁻¹)	λ_{max} (nm)	Abs	molar absorptivity (1 mol ⁻¹ cm ⁻¹)
Alizarin	240.21	8	3.3E-05	7	448	0.234	7.03E+03				448	0.234	7.03E+03			
				9	453	0.217	6.52E+03				453	0.210	6.31E+03			
				11	481	0.176	5.28E+03				498	0.173	5.19E+03			
Purpurin	256.21	8	3.1E-05	7	N/A	N/A	N/A	512	0.224	7.17E+03	N/A	N/A	N/A			
				9	507	0.238	7.62E+03	538	0.238	7.62E+03	N/A	N/A	N/A			
				11	510	0.200	6.41E+03	541	0.204	6.53E+03	N/A	N/A	N/A			
Emodin	270.24	8	3.0E-05	7	447	0.259	8.75E+03				447	0.247	8.34E+03			
				9	475	0.194	6.55E+03				478	0.194	6.55E+03			
				11	502	0.245	8.28E+03				504	0.200	6.76E+03			
Carmine	492.39	8	1.6E-05	7	506	0.115	7.08E+03				506	0.099	6.09E+03			
				9	520	0.076	4.68E+03				520	0.036	2.22E+03			
				11	529	0.081	5.00E+03				533	0.039	2.37E+03			
Luteolin	286.24	8	2.8E-05	7	372	0.519	1.86E+04				372	0.519	1.86E+04			
				9	380	0.700	2.50E+04				387	0.636	2.28E+04			
				11	382	0.591	2.11E+04				382	0.579	2.07E+04			
Apigenin	270.24	16	5.9E-05	7	342	1.211	2.05E+04				342	1.190	2.01E+04			
				9	390	1.172	1.98E+04				390	1.170	1.98E+04			
				11	393	1.482	2.50E+04				393	1.480	2.50E+04			
Kaempferol	286.23	8	2.8E-05	7	368	0.403	1.44E+04				368	1.190	2.01E+04			
				9	390	0.363	1.30E+04				390	1.170	1.98E+04			
				11	419	0.450	1.61E+04				419	1.480	2.50E+04			
Quercetin	302.236	8	2.6E-05	7	382	0.418	1.58E+04				384	0.388	1.47E+04			
				9	395	0.349	1.32E+04				392	0.220	8.31E+03			
				11	414	0.375	1.42E+04				414	0.136	5.15E+03			
Morin	302.236	8	2.6E-05	7	381	0.199	7.53E+03				381	0.183	6.90E+03			
				9	398	0.307	1.16E+04				398	0.185	6.98E+03			
				11	415	0.401	1.52E+04				415	0.262	9.89E+03			
Myricetin	318.24	8	2.5E-05	7	388	0.450	1.79E+04				385	0.396	1.57E+04			
				9	397	0.443	1.76E+04				397	0.244	9.71E+03			
				11	419	0.340	1.35E+04	660	0.162	6.44E+03	N/A	N/A	N/A	502	0.076	3.03E+03

Appendix B: Supplementary information for Chapter 5

Table B5.1 The summary of literature reviews on extraction of dyes from textiles

Sample (S): colour; date	Extraction solvent	conc	temp (°C)	t (min)	I.D	Ref.
red satin, 16 th C	few drops H ₂ SO ₄	18M		few min	ca, ka	[2]
red Ushak carpet, 17 th C:					madder	
yellow Ushak carpet, 17 th C					weld	
red Kazak carpet, 19 th C					madder	
red Kazak carpet, 19 th C					acid red azo dye	
red Angelo-Scand textile, 10-11 th C	Pyridine: W (1:1, v: v) followed by Et:H ₂ SO ₄ 10%, (3:1, v:v)		90	60	madder	[20]
red Angelo-Scand textile, 10-11 th C			90	60	maybe kermes	
some red and yellow samples					unidentified	
red Indian textiles, 16-19 th C	few drops HCl	2M	N/A	30	p, psp	[29]
blue Indian textiles, 16-19 th C					indigo	
yellow Indian textiles, 16-19 th C					unidentified	
red sample dyed with cochineal	HCl	3N	100	30	al, psp-mu, p, ru	[4]
red sample dyed with cochineal					ka, al, p	
red sample dyed with cochineal					ca, ka	
yarns dyed with coccid insect:	HCl 37%: MeOH: W (2:1:1, v/v/v)	6M	NA	10	ka, ca, fka, la	[5]
orange red Goblin tapestry, 18 th C	HCl 3N: MeOH (1:1, v/v)	1.5N	100	15	ca, car	[6]
brown red Goblin tapestry, 18 th C					ca, al, lu, ap	
light red Goblin tapestry, 18 th C					ca, al, xp	
cream Goblin tapestry, 18 th C					lu, ap, unidentified	
mustard Goblin tapestry, 18 th C					lu, ap, unidentified	
green Goblin tapestry, 18 th C					lu, in	

Table B5.1 The summary of literature reviews on extraction of dyes from textiles

Sample (S): colour; date	Extraction solvent	conc	temp (°C)	t (min)	I.D	Ref.
red textile samples from Topkapi museum, 16 th C	HCl 37%: W (1:1, v/v)	6N	20	360	la; psp, al, mu; ca	[3]
yellow Coptic textile, 7-9 th C	HCl 3N: EtOH (1:1, v/v)	1.5N	100	15	al, p (mordant Fe)	[7]
orange Coptic textile, 7-9 th C					al, p (mordant Fe, Al)	
orange Coptic textile, 6 th C					al, p, lu, ap	
red Coptic textile, 7-9 th C					al, p	
green Coptic textile, 7-9 th C					al, lu, in (mordant Fe)	
green Coptic textile, 7-9 th C					al, in (no mordant)	
black Coptic textile, 4-5 th C	HCl 3N: EtOH (1:1, v/v)	1.5N	90	20	p, kf, mu	[8]
yellow Coptic textile, 7-9 th C					p, kf	
orange Coptic textile no date					p	
black Coptic textile, 4- 5 th C	HCl 3N: EtOH (1:1, v/v)	1.5N	90	20	al, p, kf	[9]
yellow Coptic textile, 7-9 th C					al, p, lu, ap	
orange Coptic textile no date					p, lu	
light brown Scottish bog costume, 17 th C	HCl 37%: MeOH: W (2:1:1, v/v/v) followed by DMF: MeOH (1:1, v/v)	6N	100	10	lu, ap	[10]
light brown Scottish bog costume, 17 th C					ea	
brown/red Scottish bog costume, 17 th C			100	5	lu	
brown/red Scottish bog costume, 17 th C					al, p	
red Scottish bog costume, 17 th C					al, p	
orange fabric samples, 3 rd -4 th BC	HCl 37%: MeOH: W (2:1:1, v/v/v) method 1 method 1 followed by DMSO	6N	100	10	al, p, qu, lu, ap, ca, em	[11]
orange tapestry, 19 th C			100	5	al, p, xp, qu, 3,4dhab	

Table B5.1 The summary of literature reviews on extraction of dyes from textiles

Sample (S): colour; date	Extraction solvent	conc	temp (°C)	t (min)	I.D	Ref.
yellow sample dyed with pagoda tree buds	HCl37%: MeOH: W (2:1:1, v/v/v)	6N	100	10	qu	[12]
yellow sample dyed with pagoda tree buds	Formic acid: MeOH (5:95, v/v)		40	30	rut, qu, kf, kf-3,7-di-g	
yellow sample dyed with pagoda tree buds	EDTA 0.001 M: ACN: MeOH (2:10:88, v/v/v)		60	30	rut, qu, kf, kf-3,7-di-g	
yellow sample dyed with onionskins	formic and EDTA protocol				qu, qu-4'-o-g, kf	
yellow sample dyed with onionskins	HCl protocol				qu, kf	
red sample dyed with madder	3 protocols				al	
yellow sample dyed with weld	3 protocols				lu	
red sample dyed with cochineal	3 protocols				ca	
yellow sample from map, 18-19 th C	SDS (using a brush imbedded with 0.1M SDS, directly applied on the map surface)	0.1M			ga	[21]
red sample from map, 18-19 th C					ca	
brown sample from map, 18-19 th C					cr, ga	
brown sample from map, 18-19 th C					ca, ga	
blue sample from map, 18-19 th C					in	
green sample from map, 18-19 th C					cr, ga	
orange sample from map, 18-19 th C					ca, ga	
green sample from map, 18-19 th C					in	
red painting samples, 17 th C	H ₂ SO ₄	1M	25	15	al, p, qz; ca; la	[22]
	followed by: adding 2ml DCM (3 times)		vortex	10		

Table B5.1 The summary of literature reviews on extraction of dyes from textiles

Sample (S): colour; date	Extraction solvent	conc	temp (°C)	t (min)	I.D	Ref.
yellow wool dyed with weld red wool dyed with cochineal	HCl 37%: MeOH: W (2:1:1, v/v/v)	6M	100	10	lu, ap, chr ca, ka, fka	[13]
yellow sample dyed with weld pink wall paper from Chinese, 18 th C red-brown painting, 19 th C dyed with dryer's bloom dyed with dryer's bloom	ACN: MeOH (1:1, v/v) (to swell the sample) followed by adding HF followed by drying and taking up the residue into ACN: W (1:1, v/v) HCl 37%: MeOH: W (2:1:1, v/v/v)	4M 6M	25 110	30 10	lu-3,7'-di-o-glu, lu-7-O-glu, ap-7-O-glu, lu, ap la, in, ind ca, qu, br, syn bl gn-o-glu, ap-o-glu, gn, ap gn, ap	[14]
sample dyed with weld sample dyed with weld sample dyed with weld sample dyed with weld sample dyed with weld sample dyed with weld blue wool samples, 13-16 th C blue silk samples, 13-16 th C red wool samples, 13-16 th C red silk samples, 13-16 th C	HCl 37%: MeOH: W (2:1:1, v/v/v) Formic acid: MeOH: W (2:1:1, v/v/v) followed by EDTA Citric acid Oxalic acid DMSO TFA	6N 5M 0.5M 0.5M 1M 2M	100 100 100 100 100 100	15 5 5 15 15 10	lu, ap, chr, lu-7-O-glu, ap-O-glu, lu, ap, chr The same as above The same as above The same as above The same as above in, ind in, ind luc, al, p, ru al, p, in	[15]
Red silk samples, 17 th C	HCl 37%: MeOH: W (2:1:1, v/v/v)	6N	105	10	ea, ca, al, p, mu, xp, ru	[16]

Table B5.1 The summary of literature reviews on extraction of dyes from textiles

Sample (S): colour; date	Extraction solvent	conc	temp (°C)	t (min)	I.D	Ref.
red wool sample, 3 rd BC-5 th C	HCl37%: MeOH: W (2:1:1, v/v/v) and for blue and green colours HCl37%: DMSO (1:20 v/v)	6N	105	10	al,p	[17]
yellow wool from robe, 3 rd BC-5 th C		0.5M	80	5	lu	
brown sample from embroidered trousers					al	
green sample embroidered trousers					in, ind	
red silk sample from tabby					al	
red wool sample from taquete					al	
yellow wool sample from taquete					lu	
blue wool sample from taquete					in, ind	
Chinese shoes, silk robes from, 18 th & 20 th C	Formic acid: MeOH (5:95, v/v) followed by MeOH: DMF (1:1, v/v)		45-50 100	30 5	cur; ber, pal; rut, qu	[31]
yellow samples:					in, ind	
blue and dark blue					gal, ea	
brown					br; megII, fuc; ca, dcII, VII; car	
red samples						
sample dyed with cochineal	HCl37%: MeOH: W (2:1:1, v/v/v)	6N	60	30	ca, fka, ka, dc II, IV & VII	[18]
sample dyed with cochineal	Formic acid: MeOH (5:95, v/v)	5M	60	30	The same as above	
sample dyed with cochineal	TFA	2M	60	30	The same as above	
sample dyed with cochineal	Oxali cacid (0.2M): acetone:	2M	60	30	The same as above	
red silk European textile, 15 th & 16 th C	MeOH: W (0.1:3:3:4, v/v/v/v)				ca, fka, ka, dc II, IV & VII ea	
red silk European textile, 16 th C					The same as above	
red silk Turkish textile, 17 th C					The same as above	
red velvet Iranian textile, 17 th C					The same as above	
red wool Iranian rug, 19 th C					The same as above excluding ea	

Table B5.1 The summary of literature reviews on extraction of dyes from textiles

Sample (S): colour; date	Extraction solvent	conc	temp (°C)	t (min)	I.D	Ref.
Yellow, green and blue samples from Ar-raiolos carpet 18 th C	HCl 37%: MeOH: W (2:1:1, v/v/v) (method 1)	6N	100	10	lu3',7diglu, lu-7- O -glu, lu4'-O-glu, ap-7-O-glu, chr-7-O-glu, lu-3'-O-glu, ap, lu	[19]
	method 1 followed by MeOH: DMF (1:1, v/v)		100	10		
	Formic acid: MeOH 5:95, v/v)	5M	40	30		
	EDTA 0.1% in water: DMF (1:1, v/v)		60	30		
	Oxalic acid: MeOH: acetone: W: (1:30:30:40, v/v/v/v)		60	30		
	pyridine		100	15		
	DMF		100	15		
	EDTA 0.001 M: ACN: MeOH (2:10:88, v/v/v)		60	30		