Chapter 3
Results and discussion

The determination of antioxidant activity in plant extracts was developed using the free radicals DPPH and DOPH as color reagents in the FIA system. The FIA system was then coupled to an HPLC for the on-line separation and activity determination of antioxidant.

3.1 Design and optimization of the FIA system for antioxidant activity detection

In the FIA system, the analyte will react with the reagent present in the reactor. The change caused by the reaction is then measured. The choice of the reagent depends on the reaction, which will affect the sensitivity of the measurement. The reaction time is the most important parameter that has to be optimized. It depends on the length of the reactor, combined with the flow rate. Length and shape of the reactor will also affect the dispersion of the analytes.

3.1.1 Choice of test substances and reagents for the FIA

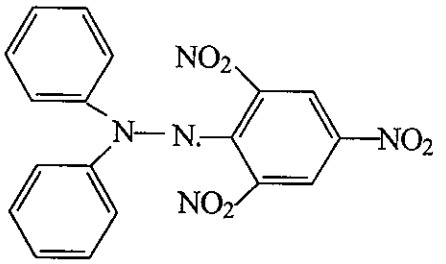
Four antioxidants i.e. rutin, quercetin, trolox and ascorbyl palmitate were used as test substances for FIA system optimization. The antioxidant activity can be measured using free radicals (R•) which can react with an antioxidant (AH):

$$R\bullet + AH \longrightarrow R\text{-}H + A\bullet$$

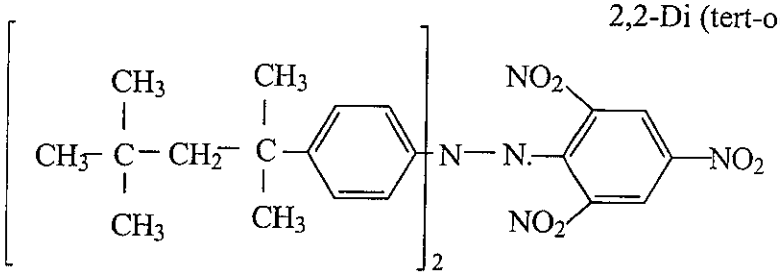
In our study, the free radicals DPPH and DOPH were used as reagents in the carrier stream. Both compounds are purple in radical forms and colorless in reduced forms. The absorption was measured at 515 or 538 nm, corresponding to the maximum absorbance of DPPH or DOPH, respectively. The decrease of the absorption correlated to the antioxidant activity of the sample.

The structures of the free radicals and antioxidant compounds are shown in Fig. 2.

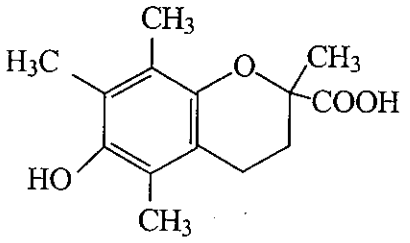
Fig. 2 The structures of the free radicals DPPH and DOPH and four antioxidants: rutin, quercetin, trolox and ascorbyl palmitate.



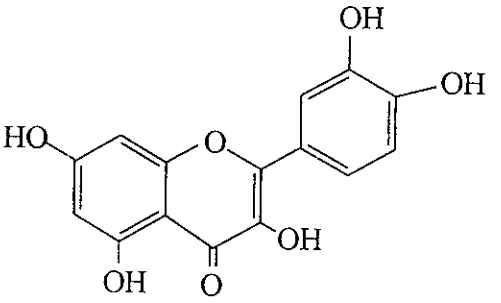
2,2-Diphenyl-1-picrylhydrazyl
(DPPH)



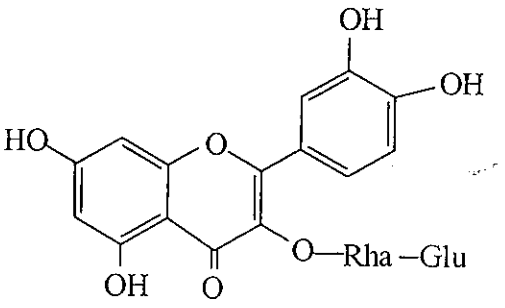
2,2-Di (tert-octylphenyl)-1-picryhydrazyl
(DOPH)



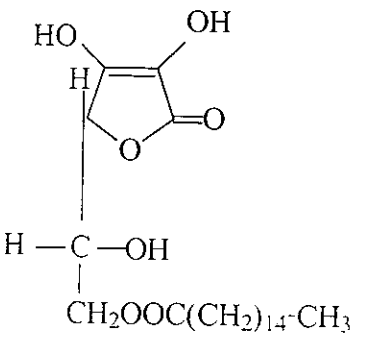
Trolox



Quercetin



Rutin



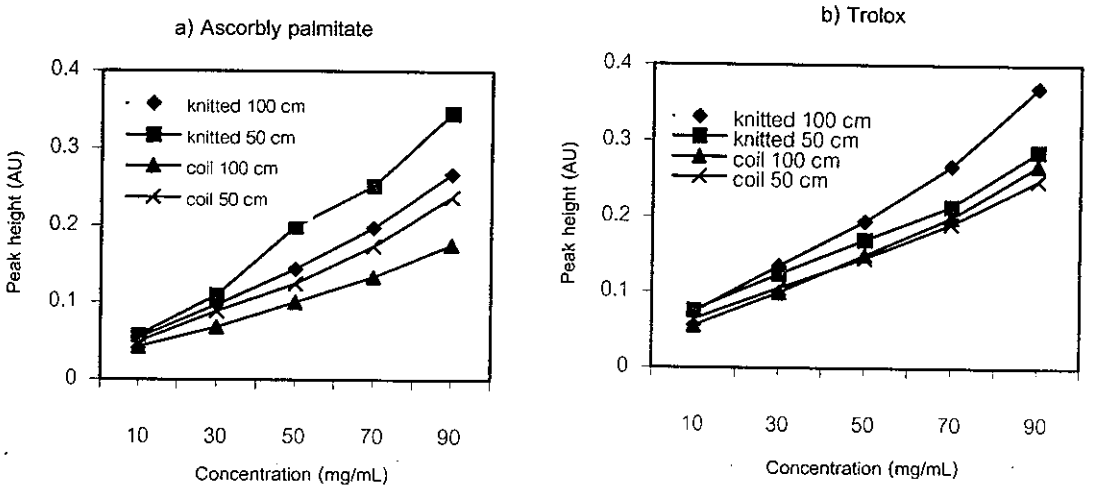
Ascorbyl palmitate

3.1.2 Shape and length of the reactor

To avoid a high degree of dispersion in the FIA system, a good mixing of analyte and reagent is required. Since the shape of the reactor affects the mixing, we compared a coiled-shape reactor with a knitted-shape reactor, both at 50 and 100 cm tubing length.

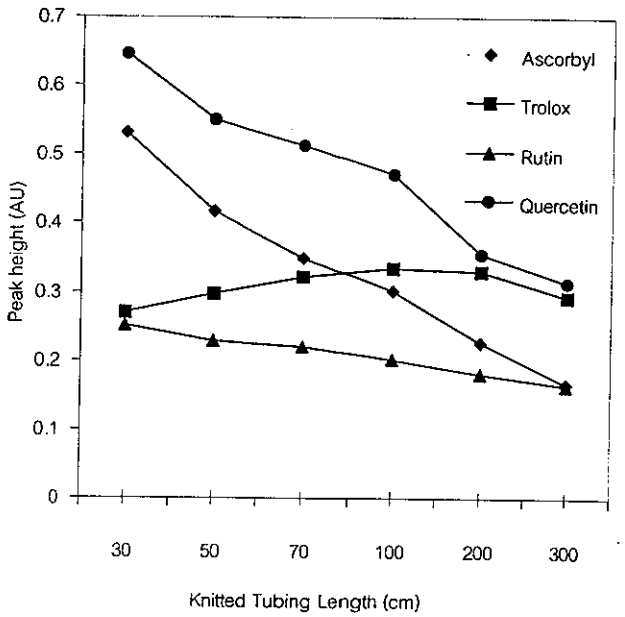
The results are shown in Fig. 3. In all cases, the knitted shape reactors gave greater peak heights indicating less band broadening comparing to that of the coiled-shape reactors.

Fig.3 Effect of the shape of the reactor on the peak heights of a) ascorbyl palmitate b) trolox. Carrier stream 0.1 mM DOPH; flow rate 0.5 ml min^{-1}; injection volume 10 µl; I.D. of reactor 0.8 mm; detection at 538 nm.



To find the optimal tubing length, the four test substances were injected into the system, using knitted-shape reactors with various lengths. The result is shown in **Fig. 4** The knitted-shape reactor with the length of 70 cm was chosen as it gave satisfactory peak heights of the four tested substances.

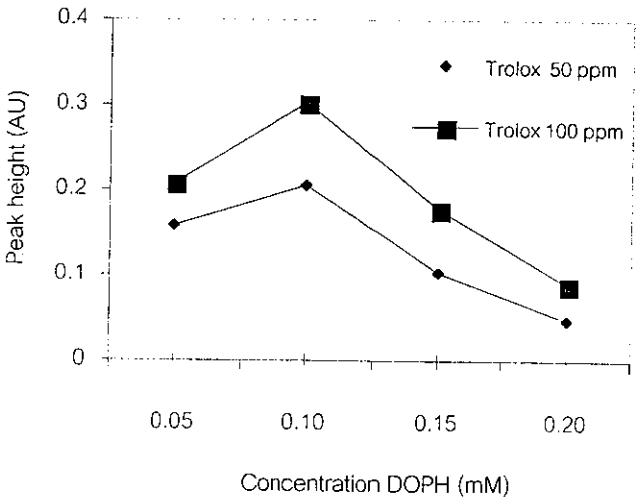
Fig.4. The peak heights of ascorbyl palmitate, trolox, rutin and quercetin when injected into FIA system with various lengths of knitted-shape reactor. Sample concentration 100 μg ml^{-1}. Other conditions as in Fig. 3.



3.1.3 Concentration of the color reagent

Various DOPH concentrations were studied. The results are shown in Fig. 5. The highest peak height was obtained when 0.1 mM DOPH was used as a color reagent in the carrier stream.

Fig. 5 The peak heights of 50 and 100 μg ml^{-1} trolox obtained from FIA using various concentrations of DOPH as a carrier stream. Other conditions as in Fig. 3.



3.1.4 Working range for quantitative analysis

After the FIA system had been developed, the system, as described in experimental, was tested for its application on quantitative analysis. The relationship between peak height and concentration of the four test compounds were investigated. The results are summarized in Table 1. For all compounds, the linear range was around 10-100 mg l^{-1}. The correlation coefficient (r) was more than 0.999 in all experiments.

Table 1 The linear ranges, limits of detection (LOD), correlation coefficient (r), and regression equation, of the four antioxidants.

Antioxidant	Linear ranges (mg l^{-1})	LOD [a] (mg l^{-1})	r	Regression equation
Rutin	10-100	5.9	0.9996	y= 0.23x+0.74
Quercetin	10-110	6.5	0.9993	y= 0.57x-2.11
Trolox	10-90	7.5	0.9991	y= 0.29x+1.30
Ascorbyl palmitate	10-100	6.5	0.9995	y = 0.604x-2.21

[a] LOD is defined as signal-to-noise ratio = 3.

3.2 HPLC coupled on-line with the colorimetric determination of antioxidant activity

In order to find the suitable conditions for the separation of two flavonoids rutin and quercetin, a C_8 analytical column, combined with a C_{18} guard column was used, and various mobile phase compositions were tested. Complete separation of the two compounds within 10 minutes was obtained using MeCN:MeOH:25 mM KH_2PO_4 pH3.0 (20:15:65 v/v/v) as a mobile phase.

For the on-line determination of the antioxidant activity after separation in HPLC, the FIA system was coupled to the HPLC by a 20 cm length of 0.064 mm I.D. of PEEK tubing. The T-connector split the column effluent into two streams with the ratio of 8:2 (v/v). The major stream went to the UV detector set at 220 nm while the minor stream went to the FIA line.

However, when the FIA reagent stream was mixed with the HPLC mobile phase mentioned above, precipitation occurred. A solubility study of 0.1 mM solution of DOPH in MeOH with various amounts of buffer using visual detection, showed that precipitation occured when the amount of the buffer exceeds 35% v/v. So, the mobile phase was then changed to 25 mM KH_2PO_4 pH3.0:MeOH 50:50 v/v. This eluent has a higher organic solvent content, but about the same eluting force. Using this mobile phase, complete separation within 8 minutes was obtained.

3.3 Optimization after coupling HPLC to FIA

Some parameters for the on-line coupling of HPLC and FIA for the separation and determination of antioxidants were varied and optimized as follows. The flow rate of HPLC was set at 1 ml min^{-1}. The sample was a solution of 500 μg ml^{-1} of rutin and quercetin in MeOH. Injection volume was 10 μl. After the column, the flow was split

into two streams; 0.8 ml min^{-1} to the UV detector, set at 220 nm, and 0.2 ml min^{-1} to the reactor. The latter one merged to DOPH, which was pumped from the FIA line.

3.3.1 Flow rate in FIA line

The various flow rates of DOPH i.e. 1.0, 0.7, 0.5 and 0.3 ml min^{-1} were tested. The FIA flow rate of 0.3 ml min^{-1} showed the highest peak height. The total flow rate in the reactor was then 0.5 ml min^{-1}.

3.3.2 Free radical reagents

Due to the poor solubility of DOPH in water, we studied the other free radical, DPPH (2,2-Diphenyl-1-picrylhydrazyl), which has a higher solubility. The standard sample solution was injected. It was found that DPPH gave higher peak heights than DOPH. Therefore, DOPH was replaced by DPPH in the further studies.

0.10 and 0.15 mM DPPH solution in MeOH were pumped through the FIA line at the previously described condition. Rutin and quercetin, at concentrations of 50 μg ml^{-1} or 500 μg ml^{-1}, were injected to the HPLC system. The results showed that 0.15 mM DPPH gave higher peak heights than 0.10 mM. However, the noise was also higher. We, therefore, selected DPPH at the concentration of 0.10 mM for the further works.

3.3.3 Split ratio

We injected□0 μl of the mixed standard antioxidants at the concentration of 500 μg ml^{-1} into the HPLC system. The ratios of the flows mobile phase split to UV detector and FIA system were varied from 8:2 to 6:4 by changing the length of the connection tubing between the HPLC and FIA system. The non-splitting system in which all of the eluate flowed through UV detection and then went to FIA system was also tested. The flow rate in the FIA line was set at 0.3 ml min^{-1}. The results showed that the non-splitting gave the lowest peak height. A split ratio of 6:4 gave the same peak height of quercetin and higher peak height of rutin when compared to that of at the ratio of 8:2. However, precipitation might occur when the DPPH mixes with high volume of HPLC mobile phase. The split ratio of 8:2 was, therefore, selected for the on-line system.

3.3.4 Reactor length

We injected the mixed standard antioxidants at the concentrations of 100 μg ml^{-1} and 50 μg ml^{-1} into the on-line system with various lengths of the reactor i.e. 50, 70, and 100 cm. A tubing length of 70 cm was selected, as it gave the highest peak heights of both flavonoids.

3.4 Detection limits

The detection limits of rutin and quercetin, defined as the amount that gives a signal to noise ratio of 3, were 500 ng and 200 ng respectively, corresponding to the concentrations of 82 and 60 μM of rutin and quercetin in the injected sample. Conditions were as described in Experimental, using the mobile phase, 25 mM

KH_2PO_4 pH 3.0:MeOH 50:50 (v/v) and DPPH as the carrier reagent. Injection volume was 20 µl.

3.5 Delay time

We injected rutin, quercetin and trolox into the developed on-line HPLC coupled with FIA system. Two chromatograms were obtained (Fig. 6). The left shows the peaks of the compounds detected at 220 nm, and the right shows their antioxidant activities with a delay time of around 50 seconds. The delay times are shown in Table 2.

Fig. 6 Chromatograms of a) 200 µg ml^{-1} of rutin (1) and 100 µg ml^{-1} of Quercetin (2), b) 100 µg ml^{-1} of trolox (3). DPPH was used as the carrier reagent. The chromatograms on the left were from HPLC-UV detector set at 220 nm. The chromatograms on the right were from FIA detector set at 515 nm; the chart speed was 2 mm min^{-1}. Other conditions as in Fig. 3.

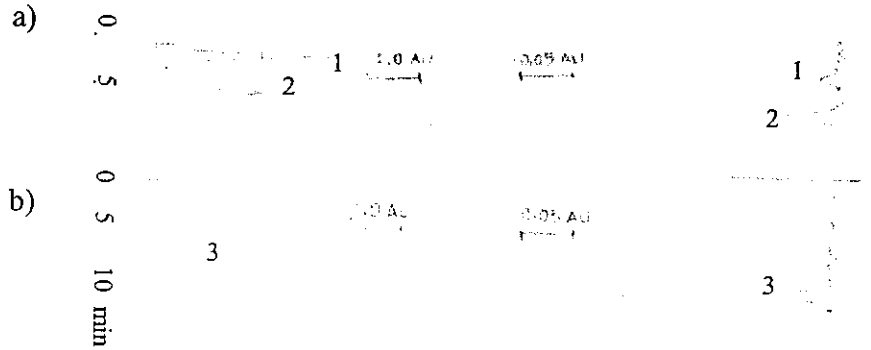


Table 2 Delay times between the HPLC-UV detector set at 220 nm and Visible detector for antioxidant activity detection set at 515nm of the HPLC coupled on-line with colorimetric detection for antioxidant activity. The results shown are the means ± S.D. of five experiments.

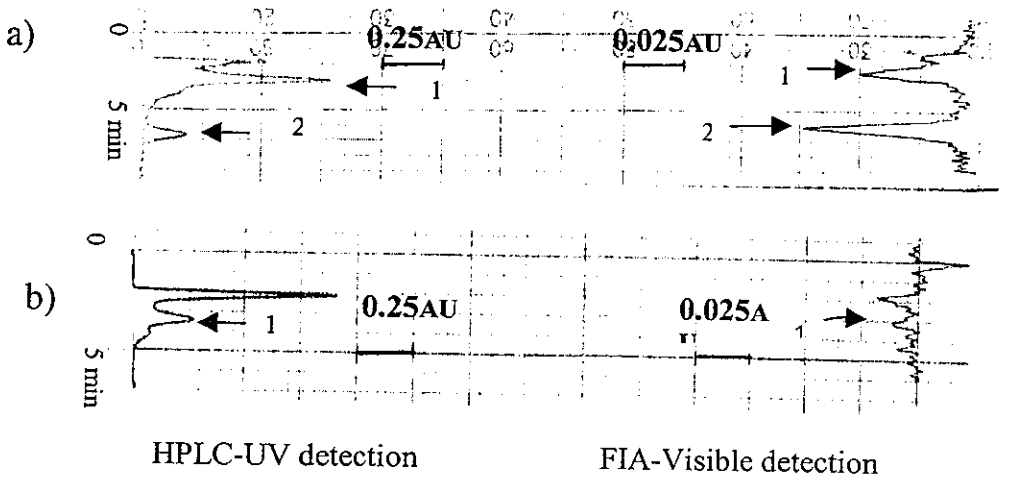
Analyte	Retention time (sec) of the analyte detected from		Delay time (sec)
	UV detector	Vis detector	
rutin	166 ± 0.005	214 ± 0.033	47 ± 0.01
quercetin	370 ± 0.012	419 ± 0.253	49 ± 0.25
trolox	435 ± 0.017	485 ± 0.028	50 ± 0.03

3.6 Applications

To show application of this method for a qualitative analysis of antioxidants in plant extracts, the extracts from flowers of *S. japonica* and leaves of *M. alba* were tested. These two plant extracts are known to contain rutin and quercetin [19, 20]. The chromatograms of two plant extracts are shown in Fig.7. The left chromatograms were obtained from the HPLC-UV detector, while the right chromatograms were

obtained from the FIA detector. The UV chromatograms show the complexity of the two extracts while there are only one or two peaks of antioxidant activity in the other chromatogram. In the analysis of the *S. japonica* crude extract, the two peaks of antioxidant activity at 213 and 419 sec correspond to the UV peak at 166 and 370 sec. They could be identified as the peaks of rutin and quercetin, respectively. In the same manner, the analysis of *M. alba* crude extract indicated the presence of rutin.

Fig.7 Chromatograms of a) 10 mg ml^{-1} of the flower of *S. japonica* L. extract b) 10 mg ml^{-1} of the leave of *M. alba* L. extract. The chromatograms on the left were from HPLC-UV detector set at 220 nm. The chromatograms on the right were from FIA-Visible detector set at 515 nm; the chart speed was 2 mm min^{-1}. Arrows 1 and 2 indicated the peaks of rutin and quercetin, respectively.



HPLC-UV detection FIA-Visible detection

This experiment demonstrates the rapid identification of the antioxidant flavonoids, rutin and quercetin from complex mixtures. This can be used as method of dereplication for these two flavonoids in drug discovery processes. By this method, rutin and quercetin can rapidly be identified in antioxidant mixtures, and excluded from further study for new antioxidants. Only sample mixtures showing unidentified antioxidant peaks should be further investigated. This method can also be used for qualitative and quantitative analysis of the two flavonoids in complex mixtures. In addition, this on-line system could be applied in rapid separation of other antioxidants from complex mixtures. The system can be further developed for on-line coupling to an identification unit such as NMR or MS. In that way, both activity determination and spectral data of the compounds isolated from HPLC will be obtained almost at the same time.