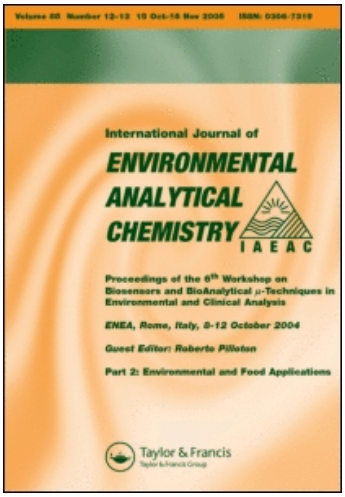

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Simultaneous determination of α-naphthol, β-naphthol and 1-hydroxypyrene in urine by synchronous fluorescence spectrometry using β-cyclodextrin as a sensitiser

Ying Wang[ab]; Yong-Sheng Wang[a]; Hong-Mei Yang[a]; Jin-Hua Xue[a]; Xuan Tan[a]; Yu Guo[a]; Xi-Lin Xiao[c]
[a] College of Public Health, University of South China, Hengyang 421001, China [b] Hengyand Blood Centre, Hengyang, 421001, China [c] College of Chemistry and Chemical Engineering, University of South China, Hengyang 421001, China

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Simultaneous determination of α-naphthol, β-naphthol and 1-hydroxypyrene in urine by synchronous fluorescence spectrometry using β-cyclodextrin as a sensitiser

Ying Wang[a,c], Yong-Sheng Wang[a]*, Hong-Mei Yang[a], Jin-Hua Xue[a], Xuan Tan[a], Yu Guo[a] and Xi-Lin Xiao[b]

[a]*College of Public Health, University of South China, Hengyang 421001, China;* [b]*College of Chemistry and Chemical Engineering, University of South China, Hengyang 421001, China;* [c]*Hengyand Blood Centre, Hengyang, 421001, China*

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A novel method for the simultaneous determination of α-naphthol (α-NAP), β-naphthol (β-NAP) and 1-hydroxypyrene (1-OHP) in human urine has been established by using synchronous fluorescence spectrometry. The measurement was carried out in sodium acetate-boroborax buffer solution (pH = 5.0) with β-cyclodextrin (β-CD) enhancing fluorescence. At $\Delta\lambda = 23$ nm, 1-OHP and β-NAP exhibit maximum signal with minimum interferences from α-NAP. At $\Delta\lambda = 175$ nm, the signal of α-NAP is not influenced by the presence of 1-OHP and β-NAP. The signals detected at these three wavelengths, 360.2 nm, 330.6 nm and 296.4 nm, vary linearly when the concentration of 1-OHP, β-NAP and α-NAP is in the range of 0.65–218.3 ng mL^{-1}, 2.8–1441.0 ng mL^{-1} and 3.6–1586.0 ng mL^{-1}, respectively. The limits of detection (LOD) for α-NAP, β-NAP and 1-OHP were 1.53 ng mL^{-1}, 0.78 ng mL^{-1} and 0.020 ng mL^{-1} with relative standard deviations (RSD) of 2.3%, 2.4% and 1.8%, respectively. The proposed method was successfully applied for the simultaneous determination of α-NAP, β-NAP and 1-OHP in human urine samples, and the obtained results were in good agreement with those obtained by the method of high-performance liquid chromatography (HPLC).

Keywords: α-naphthol; β-naphthol; 1-hydroxypyrene; synchronous fluorescence; β-cyclodextrin; double scans; urine

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants of great concern because many of them are well-known carcinogens and mutagens [1–5]. Biological monitoring is an effective way to quantitate the internal exposure to PAHs and estimate health risks due to PAHs exposure [4–6]. This was usually performed by measuring PAHs hydroxides in urine samples [3–6]. 1-Hydroxypyrene (1-OHP) is the main metabolite of pyrene and has been used as a biomarker of occupational exposure to PAHs [3–5, 7]. However, pyrene is only one kind of PAH and background urinary 1-OHP levels are influenced by many factors such as industrial pollution, cigarette smoking and diet,

*Corresponding author. Email: yongsheng.w@tom.com

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and therefore, simply using 1-OHP as a biomarker has been called into question [8,9]. Recently naphthalene was reclassified as a possible human carcinogen by several national and international committees [10]. Naphthalene and pyrene are predominant PAHs compounds in occupational exposure, whose metabolites such as α-naphthol (α-NAP), β-naphthol (β-NAP) and 1-OHP are present in urine. Several methods have been proposed for the assay of urinary naphthol [8,10,11], and 1-hydroxypyrene glucuronide [7], respectively. The simultaneous analysis of α-NAP, β-NAP and 1-OHP is usually performed mainly by using high-performance liquid chromatography method with fluorescence detection (HPLC-FD) [12,13], or gas/liquid chromatography-mass spectrometry (GC/LC-MS) [14–16]. However, these methods have several disadvantages such as necessary chromatographic separation procedure before detecting and requirement of expensive instruments. Therefore, it is significant to develop a more simple and sensitive method for the simultaneous determination of α-NAP, β-NAP and 1-OHP.

From an analytical point of view, the metabolites of PAHs have very good fluorescence properties under UV-vis excitation, and their determination can be carried out by fluorescence spectrometry. However, satisfactory analysis of a multicomponent system without any separation step is not possible when there are interferences in the spectra due to an overlap of the fluorescence bands. Fortunately, the selectivity problem resulting from the overlapping of the broad-band spectra of PAHs metabolites can be solved by using synchronous fluorescence spectrometry (SFS) method [17]. It has been known as a powerful tool for simultaneous analysis of multicomponent samples without any pretreatment of samples [18,19]. In SFS both excitation and emission monochromators are scanned simultaneously, maintaining a constant wavelength interval between them. Synchronous fluorescence technique can provide high selectivity for identification of PAHs metabolites due to the narrowing of spectral band, simplification of emission spectra, and contraction of spectral range. In recent years, SFS technique was successfully applied to biochemistry [20], drug [21], environment [22,23], food analysis [24], and so on.

So far, to our knowledge, the simultaneous determination of 1-OHP, β-NAP and 9-hydroxyphenanthrene has been reported by using one single scan technique [25]. However, no report has been found for the simultaneous determination of α-NAP, β-NAP and 1-OHP in urine by SFS method using a two-scan technique. But above all, it is necessary to select a kind of sensitising agent for enhancing fluorescence signals of α-NAP and β-NAP because they only emit weak fluorescence. β-Cyclodextrin (β-CD) is a cyclic oligomer of seven α-D-glucose units jointed by 1,4-glycosidic bonds and can be represented as a truncated cone structure with a hydrophobic cavity and hydrophilic external surface. For its special hydrophobic cavity structure, β-CD has the unique ability to form inclusion complexes with various guest molecules through non-covalent interactions [26–30]. It has been used extensively in analytical luminescence techniques for altering the photochemical and photophysical properties of the guest molecules through complexes formation, for instance, biomacromolecules [31], drug [32] and pesticides [33] were determined by using β-CD to enhance the fluorescence intensity. Therefore, we selected β-CD as a sensitising agent to enhance the fluorescence intensity of α-NAP, β-NAP and 1-OHP through the formation of inclusion complexes.

In this research, a simple, selective, sensitive and accurate method was proposed for the simultaneous determination of α-NAP, β-NAP and 1-OHP in urine samples by using β-CD as a sensitiser. In the method, two synchronous spectra were obtained by scanning both monochromators simultaneously at constant wavelength differences of $\Delta\lambda = 23$ nm for 1-OHP and β-NAP, and of $\Delta\lambda = 175$ nm for α-NAP, respectively. The metabolites of

naphthalene and pyrene are mainly excreted in urine as glucuronide and sulphate conjugates. So deconjugation of PAH conjugates of the glucuronic and the sulphuric acid is carried out by enzymatic hydrolysis with β-glucuronidase/arylsulfatase, or by acid/base hydrolysis. Thereafter, the samples were analysed directly by using the proposed method, without chromatographic separation procedure before detection. However, satisfactory analysis of α-NAP, β-NAP and 1-OHP mixture by HPLC-FD without chromatographic separation procedure is not possible due to their interferences in the spectra. Therefore, the proposed method has additional advantages of being less time-consuming, involving simple instruments and having high sensitivity. Furthermore, the recovery, selectivity and limits of detection (LOD) of this method were better than those of other constant-energy [23] and constant-wavelength synchronous fluorescence methods [34].

2. Experimental

2.1 *Apparatus*

All the fluorescence spectra were measured with a Hitachi F-4500 spectrofluorimeter (Tokyo, Japan) equipped with a 1 cm quartz cell. The slit widths of both excitation and emission were kept at 10 nm, a PMT voltage of 700 V and a response time of 0.1 s. For synchronous excitation measurements, both excitation and emission monochromators were locked together and scanned simultaneously with a constant wavelength difference $\Delta\lambda = \lambda_{em} - \lambda_{ex}$. A pH meter (PB-20, Germany) was used for pH adjustment.

2.2 *Reagents*

All experiments were performed with analytical reagent grade chemicals and doubly distilled water. For making stock solutions, 1-OHP, α-NAP and β-NAP (Sigma, purity $\geq$99%) were originally dissolved in appropriate alcohol water (1 : 1, v/v), then diluted to 100 mL with water to the final concentration of 1.00×10^{-3} mol L^{-1} for 1-OHP, 4.40×10^{-3} mol L^{-1} for α-NAP, 5.00×10^{-3} mol L^{-1} for β-NAP, respectively. The concentration of working solution for 1-OHP is 2.00×10^{-5} mol L^{-1}, and for α-NAP and β-NAP is 5.00×10^{-5} mol L^{-1}. β-CD (Shanghai Chemical Reagent Company, China) working solution (0.025 mol L^{-1}) was prepared by dissolving β-CD in 100 mL water. Sodium acetate-boroborax buffer solutions were used to control the acidity of the solution.

2.3 *Pre-treatment of urine samples*

Urine samples were collected from four cooks and two drivers. Urine specimens were pre-treated as the method reported by Ouyang *et al.* [35]. After mixing completely, a 10 mL aliquot of the urine sample was hydrolysed by 2 mL of 50% sodium hydroxide, and heated for 3.5 h in a boiling water bath away from light. After heating, the hydrolysed sample was adjusted to pH 2 $\sim$ 3 with concentrated HCl, and then filtered using a 125 mL separatory funnel. Filtrate was extracted twice with 6 mL of n-hexane, and the organic phase was evaporated to dryness at 30°C under a gentle flow of nitrogen gas. The residue was redissolved in 0.2 mL of alcohol water (1 : 3, v/v) and analysed directly with the developed method.

Several different extracting solvents for urine samples, such as dichloromethane, chloroform, benzene, diethyl ether, petroleum ether and n-hexane, were tested. Taking the extraction efficiency and foreign substance interference into account, n-hexane was selected for the extraction of 1-OHP, α-NAP and β-NAP in hydrolysed samples.

2.4 *Procedure*

In a series of 5 mL test tubes: 2.2 mL of 0.025 mol L^{-1} β-CD was mixed with 1.2 mL of sodium acetate-boroborax buffer solutions (pH 5.0), an appropriate amount of 1-OHP or/and α-NAP and/or β-NAP or the sample solutions, which were diluted to 5 mL with doubly distilled water. After shaking, the synchronous fluorescence spectra were obtained by scanning both monochromators simultaneously at constant wavelength differences of $\Delta\lambda = 23$ nm for 1-OHP and β-NAP, and of $\Delta\lambda = 175$ nm for α-NAP, respectively. The synchronous fluorescence intensities were measured at the synchronous maxima of each compound: for 1-OHP $\lambda_{max} = 360.2$ nm, for β-NAP $\lambda_{max} = 330.6$ nm, for α-NAP $\lambda_{max} = 296.4$ nm, respectively.

3. Results and discussion

3.1 *Spectral characteristics*

The determination for metabolites of PAHs can be carried out by fluorescence spectrometry. However, the conventional fluorescent analysis of multi-component system suffers from the overlapping of peaks of interest. Figure 1 showed the fluorescence emission maximum of α-NAP (5.0×10^{-6} mol L^{-1}, curve 1), β-NAP (5.0×10^{-6} mol L^{-1}, curve 2) and 1-OHP (5.0×10^{-7} mol L^{-1}, curve 3) was located at 476.6 nm, 352 nm and 388 nm, respectively. It was noticeable that the fluorescence spectra of α-NAP, β-NAP and 1-OHP were overlapped largely, because their structures are very similar. Therefore, the analysis of these metabolites would not be feasible by conventional spectrofluorometry at their wavelength maxima. Nevertheless, it can be seen from Figure 1 that although their

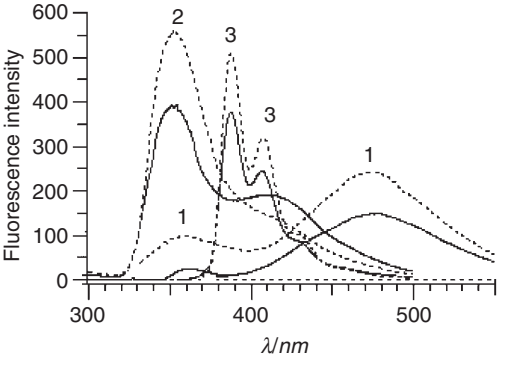

Figure 1. Fluorescence spectra of α-NAP ($\lambda_{ex} = 293$ nm), β-NAP($\lambda_{ex} = 273$ nm) and 1-OHP ($\lambda_{ex} = 343$ nm).
Note: 1. α-NAP (5.0×10^{-6} mol L^{-1}); 2. β-NAP (5.0×10^{-6} mol L^{-1}); 3. 1-OHP. (5.0×10^{-7} mol L^{-1}); ——: in the absence of β-CD,: ……: in the presence of β-CD; the concentration of β-CD is 0.011 mol L^{-1}.

fluorescence spectra were overlapped, the patterns were clearly different, and there was a bigger wavelength interval between their fluorescence emission maxima. Therefore, the interferences in the spectra due to an overlap of the fluorescence bands can be overcome by using SFS method mainly based on narrowing of spectral band, simplification of emission spectra, and contraction of spectral range. The synchronous spectra of 1-OHP, β-NAP and α-NAP were obtained by maintaining a constant wavelength differences of $\Delta\lambda = 23$ nm for 1-OHP and β-NAP, and of $\Delta\lambda = 175$ nm for α-NAP (Figure 2), respectively. As shown in Figure 2(a), the peaks corresponding to 1-OHP and β-NAP were well resolved and the maximum peaks were at 360.2 nm for 1-OHP and 330.6 nm for β-NAP, respectively. From Figure 2(b), the signal of α-NAP is not influenced by the presence of 1-OHP and β-NAP and the maximum fluorescent signal was at 296.4 nm. So it is possible to determine three metabolites of PAHs simultaneously in a mixture by using two scans. Furthermore, it can be easily observed that β-CD can enhance notably fluorescence intensity (Figure 1). It also can be seen from Figure 2 that the synchronous fluorescence peaks of 1-OHP, β-NAP, α-NAP and their mixture solution were correlated well at $\lambda = 360.2$ nm for 1-OHP, $\lambda = 330.6$ nm for β-NAP and $\lambda = 296.4$ nm for α-NAP. Therefore, the corresponding peaks were chosen for the determination of 1-OHP, β-NAP and α-NAP, and a synchronous fluorescence spectrometric method was developed.

3.2 *Optimisation of experimental conditions*

3.2.1 *Selection of optimum* $\Delta\lambda$

In synchronous fluorescence technique, $\Delta\lambda$ can directly influence fluorescence intensity, spectral shape and bandwidth. Moreover, the $\Delta\lambda$ which gives the highest synchronous fluorescence intensity for a particular species is chosen as the optimum $\Delta\lambda$ and the corresponding peak is taken as the optimum peak. Therefore, a suitable $\Delta\lambda$ value is needed for the accurate determination of each component in a complex sample [19,22]. The selection of this parameter was usually made empirically to achieve maximum selectivity for each component signals in a mixture. To find the most suitable $\Delta\lambda$ value for the precise determination of 1-OHP, β-NAP and α-NAP, a wide range of $\Delta\lambda$ values ($5 \sim 190$ nm) were examined. As can be seen from Figure 2(a), the maximum fluorescence intensities of

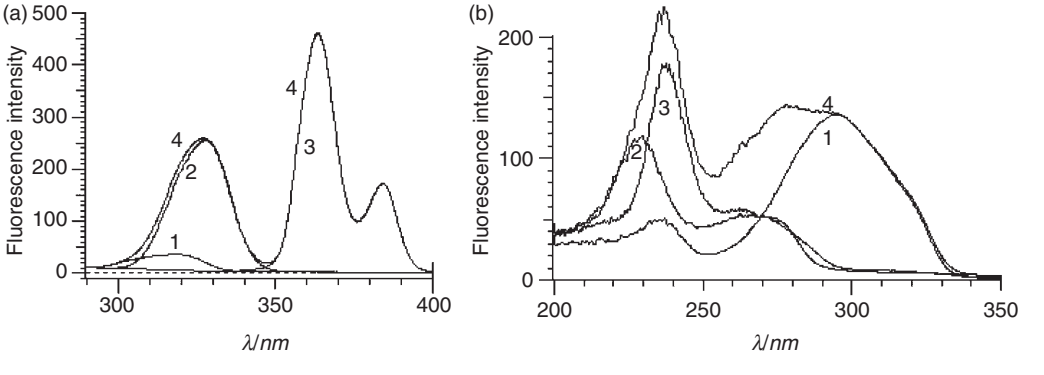

Figure 2. Synchronous fluorescence spectra of (a) β-NAP and 1-OHP at $\Delta\lambda = 23$ nm and (b) α-NAP at $\Delta\lambda = 175$ nm.
Note: 1. α-NAP; 2. β-NAP; 3. 1-OHP; 4. their mixture; the concentrations of α-NAP, β-NAP, 1-OHP and β-CD are the same as those in Figure 1.

1-OHP and β-NAP correspond to the wavelength interval (Δλ) 23 nm. So the Δλ of 23 nm is an ideal value for the quantification of 1-OHP and β-NAP because it gave a narrower single spectrum with the highest selectivity, and displayed a minimum interference of α-NAP. The synchronous spectra at 360.2 nm and 330.6 nm correspond to 1-OHP and β-NAP at $\Delta\lambda = 23$ nm, and SFS of the mixture of 1-OHP and β-NAP correlated well with that of their individual solution at 360.2 nm and 330.6 nm, respectively. Figure 2(b) showed that α-NAP exhibits maximum signal with minimum interferences from 1-OHP and β-NAP when Δλ was fixed at 175 nm, and the maximum synchronous peak of α-NAP was located at 296.4 nm, which correlated well with that of α-NAP individual solution. Therefore, the corresponding peaks were chosen for the quantification of 1-OHP, β-NAP and α-NAP, respectively.

3.2.2 *Effect of pH*

The effect of solution pH on the synchronous fluorescence intensity was investigated. Figure 3(a) showed that the fluorescence intensity reached their maximum and remained constant in the pH range of 3.0 ~ 8.0 for 1-OHP and β-NAP, and of 3.0 ~ 9.0 for α-NAP. Besides, the fluorescence intensity of 1-OHP, α-NAP and β-NAP decreased rapidly with increasing pH above 9. One possible reason was that pKa was 7.4 for 1-OHP [35], 9.4 for α-NAP [36] and 9.5 for β-NAP [32], at pH < 8.0, these three metabolites existed mainly as neutral molecule (ArOH), when pH > 8.0, neutral molecule ArOH decreased and the form of anion (ArO^-) increased gradually. It can be demonstrated in Figure 3(b) that with increasing of pH, the fluorescence intensity of β-NAP at 330.6 nm gradually decreased along with a growth of a new fluorescence peak at 362 nm, which enhanced gradually with increasing pH. So, sodium acetate-boroborax buffer solution of pH 5.0 was selected to control the acidity of the solutions.

3.2.3 *Effect of β-CD concentration*

The influence of β-CD concentration in the range of 0 ~ 0.015 mol L^{-1} on the fluorescence intensity of α-NAP was examined in sodium acetate-boroborax buffer solutions (pH 5.0). It can be seen in Figure 4 that α-NAP only emits relatively weak fluorescence in the

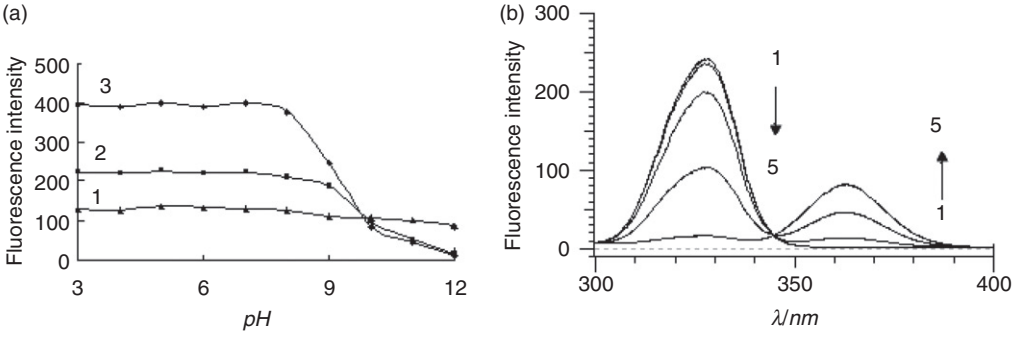

Figure 3. Effect of pH on synchronous fluorescence of (a) α-NAP, β-NAP and 1-OHP and (b) β-NAP.
Note: 1. α-NAP; 2. β-NAP; 3. 1-OHP; the concentrations of α-NAP, β-NAP, 1-OHP and β-CD are the same as those in Figure 1; (b) pH from 1 to 5: 5.0; 7.0; 9.0; 11.0; 12.0.

absence of β-CD. With increasing of β-CD concentration, the fluorescence intensity was markedly enhanced and reached the maximum when β-CD concentration is 0.011 mol L^{-1}. The same results were also obtained when 1-OHP or β-NAP was examined (data not shown). Therefore, the β-CD with the concentration of 0.011 mol L^{-1} was chosen for the further experiments.

It was demonstrated that the sensitising effect of β-CD was probably due to the formation of inclusion compounds [26,31]. As shown in Figure 1, the fluorescence intensities of α-NAP, β-NAP and 1-OHP were markedly enhanced in the presence of β-CD. These changes suggest not only the formation of β-CD-α-NAP, β-CD-β-NAP or β-CD-1-OHP complexes but also the big changes of the microenvironment around the guest molecules. In these complexes, α-NAP, β-NAP or 1-OHP are held within the cavity of β-CD molecule. The lipophilic cavity of β-CD molecule provides a microenvironment, into which non-polar moieties of α-NAP, β-NAP or 1-OHP with appropriate size can fit and form inclusion complexes [26,37]. So, the formation of inclusion compound is a dimensional fit between β-CD cavity and α-NAP, β-NAP or 1-OHP guest molecule. When α-NAP, β-NAP or 1-OHP was entrapped in the β-CD cavity, this microenvironment with stronger rigidity would restrict the freedom of α-NAP, β-NAP or 1-OHP. Moreover, the steric hindrance of β-CD torus can protect the excited states from non-radiative and quenching processes, which normally readily occur in aqueous solution [26]. These factors could increase fluorescence quantum yield and enhance the fluorescence efficiencies of α-NAP, β-NAP or 1-OHP molecules.

3.3 *Analytical parameters*

The calibration graph for the determination of 1-OHP, α-NAP and β-NAP was described under the optimum conditions. The results showed that there was a linear relationship between the fluorescence intensity and the concentration of 1-OHP, β-NAP and α-NAP in the range of 0.65–218.3 ng mL^{-1}, 2.8–1441.0 ng mL^{-1} and 3.6–1586.0 ng mL^{-1}, respectively. The linear regression equations of calibration graph were $\Delta F_{1\text{–OHP}} = 3.5\rho(\text{ng mL}^{-1}) - 1.8$, $\Delta F_{\beta\text{–NAP}} = 0.32\rho(\text{ng mL}^{-1}) + 1.2$ and $\Delta F_{\alpha\text{–NAP}} = 0.18\rho(\text{ng mL}^{-1}) + 1.7$ with a correlation coefficient of $r_{1\text{–OHP}} = 0.9995$, $r_{\beta\text{–NAP}} = 0.9998$ and $r_{\alpha\text{–NAP}} = 0.9998$, respectively.

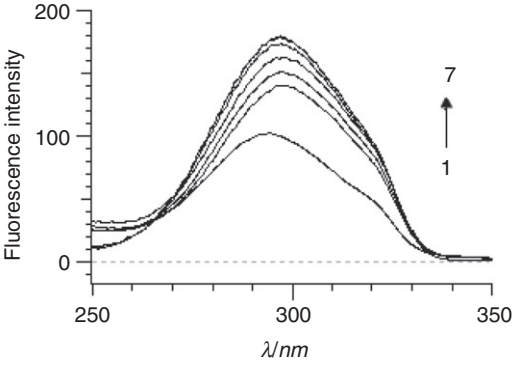

Figure 4. Effect of β-CD concentration on the fluorescence intensity of α-NAP.
Note: α-NAP (8.0×10^{-6} mol L^{-1}); pH = 5.0; the concentration of β-CD from 1 to 7 (mmol L^{-1}): 0; 0.25; 0.5; 1.0; 5.0; 11.0; 15.0.

According to the IUPAC definition [38], the limit of detection (LOD) is 0.020 ng mL^{-1} for 1-OHP, 0.78 ng mL^{-1} for β-NAP and 1.53 ng mL^{-1} for α-NAP. These results were calculated with the equation $LOD = 3S_b/m$ [33,39], where S_b is the standard deviation of the blank measurements ($n = 11$) and m is the slope of the calibration graph. The limit of quantitation (LOQ) is 0.060 ng mL^{-1} for 1-OHP, 2.6 ng mL^{-1} for β-NAP and 5.1 ng mL^{-1} for α-NAP, obtained by the formula $LOQ = 10S_b/m$. The relative standard deviations for the nine determinations of 0.11 ng mL^{-1} of 1-OHP, 0.14 ng mL^{-1} of β-NAP and 0.14 ng mL^{-1} of α-NAP were 1.8%, 2.4% and 2.3%, respectively. The proposed method has a number of advantages over other methods, such as better sensitivity, wide linear ranges of determination, higher resolution and higher recoveries [23,34]. Furthermore, this method is more convenient and faster than HPLC because the sample can be analysed directly without chromatographic separation procedure before detection [8,13].

3.4 *Tolerance of foreign substances*

In order to assess the effects of foreign substances on the synchronous spectrofluorimetric procedure described above, the selectivity of this assay was studied by analysing synthetic sample solutions containing 44 ng mL^{-1} of 1-OHP, 280 ng mL^{-1} of α-NAP and β-NAP respectively, where a series of other ions and organics had been added. The tolerance level was defined as an error not exceeding $\pm 5\%$ in the determination of the analytes. The allowable quantities (mg) of the following ions or matters were respectively: Na^+(5.6), K^+(0.6), $C1^-$(0.9), Fe^{3+}(0.02), Fe^{2+}(0.01), NH_4^+(0.015), Ba^{2+}(0.5), Ca^{2+}(0.56), I^-(0.61), F^-(0.49), NO_3^-(0.45), NO_2^-(0.4), HCO_3^-(0.03), urea (0.5), SO_4^{2-}(5.3), HPO_4^{2-}(0.74), alcohol (0.52), glucose (0.49), guanine (0.08), citric acid (0.04), n-hexane (0.3), BSA (0.01), methanol (0.4), hydroxybenzene (0.02), trichlormethane (0.05), petroleum ether (0.07), and diethyl ether (0.05). It was observed that common metal cations, inorganic anions and many organics were tolerated at high concentration; however, proteins such as bovine serum albumin show poor tolerance. Because protein content is very low in human urine and removable by urinary pyrohydrolysis, therefore, the proposed method exhibits good selectivity.

3.5 *Analytical application to urine samples*

Under the experimental condition, the proposed method was applied to the determination of 1-OHP, α-NAP and β-NAP in six urine samples from four cooks and two drivers. Recovery test was carried out by adding a known amount of 1-OHP, β-NAP and α-NAP to the urine samples, respectively. The results gained from the synchronous fluorescence spectrometry and those from the HPLC method were compared. A good comparability between these results obtained with HPLC and SFS was shown in Table 1. It is obvious that the proposed method for the simultaneous determination of 1-OHP, β-NAP and α-NAP was reliable, sensitive and practical.

4. Conclusion

The proposed method showed that synchronous spectrofluorimetry using a two-scan technique is a useful approach for resolving a α-NAP, β-NAP and 1-OHP mixture. It has been applied successfully for the simultaneous determination of α-NAP, β-NAP and

Table 1. Analytical results for human urine samples (n = 6).

Samples	Substances	Original μg L^{-1}	RSD %	Added μg L^{-1}	After added μg L^{-1}	Recovery %	HPLC μg L^{-1}	t
1	α-NAP	50.7	2.8	28.8	81.9	103	51.2	0.94
	β-NAP	39.9	3.3	28.8	64.6	94.0	38.9	1.90
	1-OHP	2.42	4.2	4.37	6.48	95.4	2.18	1.34
2	α-NAP	49.2	2.5	28.8	74.9	96.0	48.1	0.89
	β-NAP	30.0	3.9	28.8	61.9	105	28.9	1.75
	1-OHP	1.70	3.8	4.37	5.80	95.6	1.87	1.97
3	α-NAP	53.3	1.3	28.8	86.5	105	51.2	0.71
	β-NAP	65.6	1.5	28.8	89.3	94.6	67.9	1.53
	1-OHP	3.20	3.3	4.37	7.59	100	3.07	2.12
4	α-NAP	73.5	2.2	57.7	123	93.5	71.9	1.21
	β-Nap	61.9	1.6	57.7	125	104	63.1	0.87
	1-OHP	3.80	1.8	8.73	13.0	104	3.98	0.97
5	α-Nap	78.9	1.5	57.7	130	94.9	79.8	0.98
	β-NAP	89.2	3.4	57.7	141	95.9	88.1	1.23
	1-OHP	4.40	1.9	8.73	13.7	105	4.21	0.79
6	α-NAP	80.7	1.4	57.7	130	94.2	83.1	1.35
	β-NAP	87.1	2.5	57.7	147	101	85.3	0.89
	1-OHP	5.30	2.3	8.73	13.5	96.4	5.14	1.81

1-OHP in human urine without chromatographic separation procedure before detection, which are in a good agreement with those obtained by HPLC. Furthermore, the proposed method is found to have a lot of advantages such as being less time-consuming, involving simple instruments, and having high sensitivity and low interference. The recovery and limits of detection (LOD) of this method were better than that obtained by other synchronous fluorimetric methods. Although SFS methods are sensitive, selective and rapid for multi-component analysis, they fail for very complex mixtures where there is a spectral loss as a result of the interference of the different species in the sample. Therefore, more in-depth research is still required in both fundamental and applied aspects for progress in this area to be made in the future.

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