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Detection of flunitrazepam through photocatalytic reaction of ZnO particles in coloured spirits by UV-Vis spectrophotometer

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ABSTRACT: The photocatalytic effect of ZnO particles was developed for the detection of standard flunitrazepam and Rohypnol tablets in coloured spirit solution by a UV-Vis spectrophotometer. It is a screening technique for qualitative detection and quantitative estimating flunitrazepam concentration before getting the precise values by other high performance techniques. In an alcoholic solution with ZnO particles, the flunitrazepam is changed to 7-amino-flunitrazepam after irradiation. The absorption band shifts to a longer wavelength (309 nm to 345 nm). The photocatalytic parameters such as the reaction temperature, ZnO concentration, and irradiation time were studied in this work. With the optimum condition, the detection limits of standard flunitrazepam and Rohypnol are 0.024 mg/l and 0.092 mg/l in a solution mixture of ethanol and soda water (1:25). The method was applied to detect the flunitrazepam in coloured spirit brands in Thailand.

KEYWORDS: Rohypnol, photo-catalysis, 7-amino-flunitrazepam

INTRODUCTION

Flunitrazepam is an *N*-methyl-2′-fluoro analogue of nitrazepam with a molecular mass 313.3 g/mol. Commonly, flunitrazepam is manufactured by the pharmaceutical company Hoffmann-La Roche Inc.[1] under the trade name "Rohypnol" and commonly known as "roofies"[1–6]. Flunitrazepam has been particularly clinically used in the short-term treatment of sleep disturbances as a sedative and as a pre-anaesthetic medication in intensive care. Flunitrazepam also has a special capacity for provoking violence and aggression. It can impair judgement and motor skills and cause memory loss or blackouts[5–7]. A person under the influence of flunitrazepam can appear to be drunk, display lack of coordination, blood-shot eyes, and slurred speech. Flunitrazepam is a colourless, tasteless, and odourless pill which is easily ground down into powder[8,9]. Consequently, Rohypnol tablets include a colour additive to be more easily detected[10]. It is soluble in alcohol but insoluble in water. At the present time, it is abused in the area of drug-facilitated sexual assaults because the victims are unable to clearly recall the assault as a result of marked anterograde amnesia[1,6]. In the US, flunitrazepam has been labelled a "club drug" by the National Institute on Drug Abuse[8,11]. Many efforts have been researched to detect the flunitrazepam by indirect laser-induced fluorescence detection on a microfluidic device[12], GC/MS, or HPLC from specimen testing: body fluid, hair, etc.[13–22] Although the detection of flunitrazepam by these techniques is a precise quantitative analysis, the sample preparation is complicated, time consuming, and expensive. It would be an advantage to screening flunitrazepam readily from alcoholic drinks by a simple technique like UV-Vis absorption spectrophotometry owing to its convenient sample preparation, low cost, and fast detection. One Rohypnol tablet (1 mg) is usually put directly into a glass of spirit and mixed with soda water. However, it is very difficult to observe the changing of its colour in coloured spirits.

In recent years, heterogeneous photocatalytic processes were used in the bleaching of wine and coffee traces by photodegradation process of metal oxide[23]. It has inspired us with the idea of using this method in detecting flunitrazepam in coloured spirits.

In this work, we report the simple method to detect flunitrazepam in coloured spirits by using a UV-Vis spectrophotometer. The absorption band of coloured spirits occurs in the range of 250–345 nm, hindering the absorption bands of flunitrazepam samples at 253 and 304 nm. We aim to bleach the colour of the spirit by using UV irradiation of ZnO particles via photocatalytic process. Flunitrazepam absorption bands are proposed to be detected. In this report, results were obtained from irradiating the standard and sample flunitrazepam (Rohypnol) both in the mixture of ethanol and soda water and alcoholic beverages (Regency, 100 Pipers, and Master blend) in order to simulate the real situation of flunitrazepam exposure. The optimum conditions like temperature effect, irradiation time, and amount of ZnO of the irradiation were studied.

MATERIALS AND METHODS

Materials

Standard flunitrazepam was obtained from the Regional Forensic Science sub-Division 41, Office of Police Forensic Science, Royal Thai Police. Rohypnol drug was purchased from Hat-Yai hospital legally under the permission from the Ministry of Public Health of Thailand. ZnO, AR grade, was purchased from Fluka.

Instruments

The UV-Vis absorption spectra were recorded with a Shimadzu UV-160A spectrophotometer using standard quartz cells. Electron ionization mass spectra were recorded on an MAT 95 XL Mass Spectrometer, Thermofinnigan. The samples were irradiated under blacklight fluorescence 3×15 W in a dark chamber.

Optimum condition of ZnO photocatalytic process

The 40 mg/l stock solutions of standard flunitrazepam were prepared in ethanol by dissolving 1 mg of standard flunitrazepam in 25 ml of ethanol. The analytical methods were generated from the 1.5 mg/l standard flunitrazepam in ethanol adjusting the volume to 10 ml by soda water. Experimental effort was spent on exploring the optimum ZnO amount, irradiation time, and temperature.

Amount of ZnO

0.05, 0.08, and 0.10 g of ZnO powder were added in each set of 10 ml of 1.5 mg/l standard flunitrazepam solution and then transferred into a capped glass tube in order to prevent the solvent evaporation. The glass tubes were irradiated using 3×15-W blacklight fluorescent tubes and continuously stirred at 45 °C for 60 min. After that, the mixtures were collected and centrifuged to separate the ZnO powder. The clear solutions were measured for the absorption spectra.

Irradiation time

(0.08 g) ZnO powder was added in each set of 10 ml of 1.5 mg/l standard flunitrazepam solution and then transferred into a capped glass tube. It was irradiated using 3×15-W blacklight fluorescent tubes and continuously stirred at 45 °C for 15, 30, and 60 min. After that, all samples were collected and centrifuged to separate the ZnO powder. The clear solutions were measured for the absorption spectra.

Effect of temperature

(0.08 g) ZnO powder was added in each set of 10 ml of 1.5 mg/l standard flunitrazepam solution and then transferred into a capped glass tube. It was irradiated using 3×15-W blacklight fluorescent tubes and continuously stirred at room temperature for 30 min. After that, all samples were collected and centrifuged to separate the ZnO powder. The clear solutions were measured for the absorption spectra. In the same manner, samples were prepared and measured at 35, 50, 65, 80, and 95 °C, respectively.

Calibration curves

Various concentrations (0.05–1.25 mg/l) of standard flunitrazepam and Rohypnol were prepared in 10 ml of the mixture of ethanol and soda water in capped glass tubes. The Rohypnol stock solution can be prepared by dissolving a Rohypnol tablet (1 mg) in 25 ml of ethanol and stirring for 5 h. The Rohypnol solution was filtered to remove the insoluble drug additives which are lactose, microcrystalline cellulose, ethyl cellulose, sodium starch, talc, TiO_2, and iron oxide. The absorption spectra were measured. ZnO (0.08 g) was added into both standard flunitrazepam and Rohypnol solutions. The samples were irradiated under blacklight fluorescent tubes and continuously stirred at 80 °C for 30 min. The solutions were separated by using a centrifuge. The clear solutions were measured for the absorption spectra and compared the results before and after irradiation.

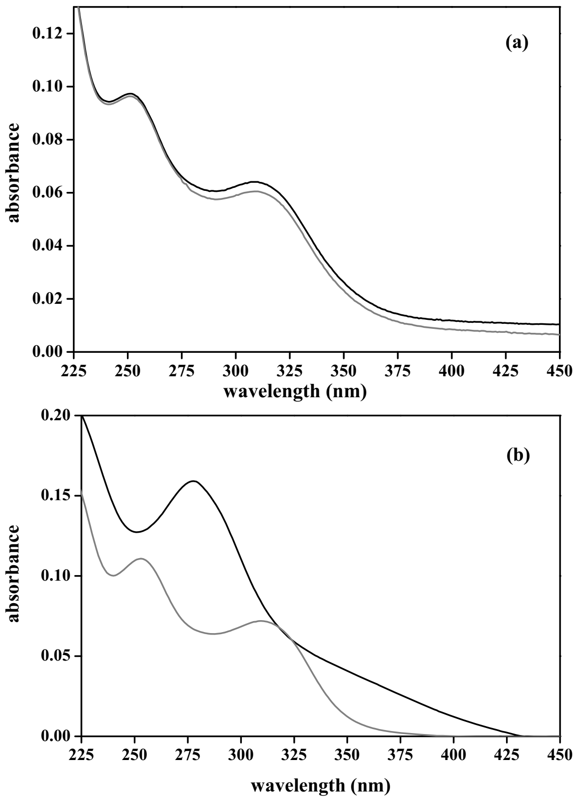


Fig. 1 (a) Absorption spectra of 2 mg/l flunitrazepam standard solution (black line) and Rohypnol solution (grey line). (b) Absorption spectra of a sample of distributed coloured spirit solution (black line) and Rohypnol solution (grey line).

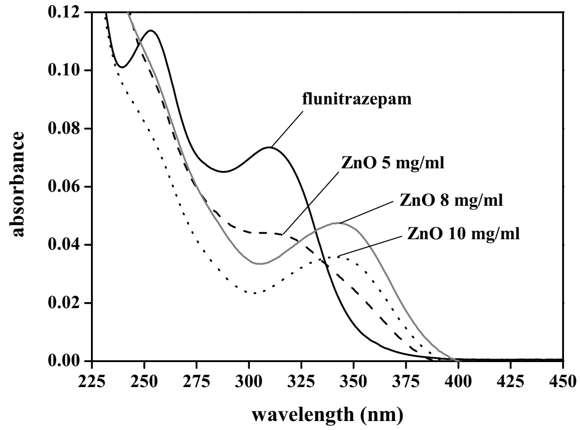


Fig. 2 Effect of ZnO concentration in 1.5 mg/l of flunitrazepam standard solution at 45 °C after irradiation time for 1 h.

RESULTS AND DISCUSSION

Absorption spectra of flunitrazepam in ethanol and coloured spirits

The absorption spectra of 2 mg/l (2 ppm) standard flunitrazepam and Rohypnol solutions in a mixed solution of ethanol and soda water are shown in Fig. 1a.

There are two absorption bands which appear at the maximum wavelength of 253 and 309 nm in the UV region. Both bands arise from the π-π* transitions with molar extinction coefficient $\sim 10^4$ $M^{-1}cm^{-1}$. The Rohypnol absorption spectrum is slightly shifted from that of flunitrazepam solution with no significant difference (Fig. 1a and Table 1). The interference from the matrix in Rohypnol tablet can be neglected. The shifting may occur from the additive of drug composite like the colouring agent, lactose, and powder. The detection of flunitrazepam species in the coloured spirit cannot be done simply. The absorption band of the spirit itself hinders the absorption band of flunitrazepam as exhibited in Fig. 1b.

Effect of photocatalytic reaction parameters

The photocatalytic activity of bare ZnO catalyst was evaluated by measuring the changing of absorption spectra of the flunitrazepam standard solution of ethanol and soda water. The concentration ratio between ethanol and soda water did not affect the absorption spectra of flunitrazepam because the absorption cut-off for ethanol and soda water is below 240 nm which does not affect the absorption band of flunitrazepam (345 nm) after irradiation. In addition, absorption spectrum of each concentration of flunitrazepam has been deducted by the absorbance of each being blank with the relative ratio between ethanol and soda water. The effect of photocatalytic reaction parameters was first studied in order to determine the most suitable condition. In general, the rate of heterogeneous photocatalytic reaction usually depends on many parameters but, in this work, irradiation time, amount of ZnO particles, and reaction temperature were concerned.

When the irradiation time was fixed at 1 h and reaction temperature was first selected at 45 °C, the appearance of this band is affected by the amount of ZnO particles (Fig. 2).

The rate of photocatalytic reaction was increased by increasing ZnO particles. At ZnO concentration of 5 mg/ml, a broad band presented at $\lambda_{\max} \sim 320$ nm arising from an incomplete reaction. It was found that the 8 mg/ml of ZnO was an optimum amount. The reaction was completed and the band at $\lambda_{\max} \sim 345$ nm appeared in the absorption spectrum. The ZnO amounts over 8 mg/ml decreased the absorbance at 345 nm because the catalyst was already working at

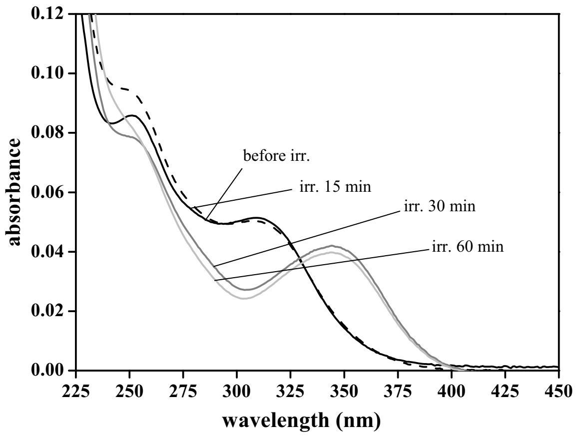


Fig. 3 Absorption spectra of 1.5 mg/l of flunitrazepam standard solution with ZnO = 8 mg/ml at various irradiation times.

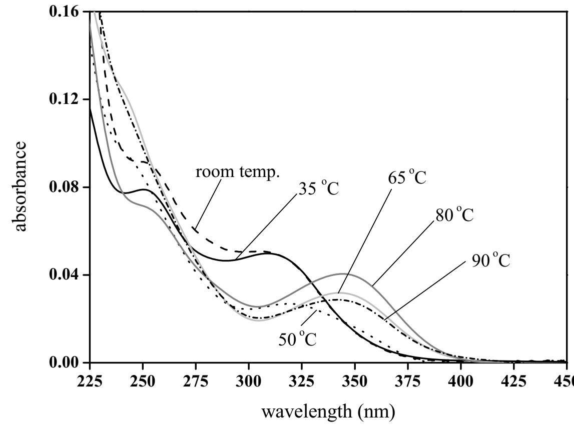


Fig. 4 Absorption spectra of 1.5 mg/l of flunitrazepam standard solution with 8 mg/ml after irradiation time = 30 min at various reaction temperatures.

its maximum capacity. In addition, too high catalyst concentration leads to the hindrance regarding their photon absorption. Generally, many compounds are adsorbed by metal oxide particles and then react with the reactive species generated from the photocatalytic reaction on the metal oxide surface before transforming to other species. Unless the ZnO particles absorb the photon, the flunitrazepam will be only adsorbed on the ZnO surface. This phenomena reduces the intensity of the band at $\lambda_{\max} \sim 345$ nm with high ZnO concentrations.

Fig. 3 shows the changing of absorption spectra of flunitrazepam standard solution at various irradiation times. After 30 min of irradiation, a new absorption band at $\lambda_{\max} \sim 345$ nm was presented while the previous one at $\lambda_{\max} \sim 309$ nm had disappeared. However, it showed no significant difference between absorption spectra of flunitrazepam standard solution after irradiated for 30 min and 60 min.

Another parameter increasing the rate of reaction is temperature. In this try, the effect of reaction temperature was carried out between 35–95 °C with 30 min irradiation time. The photocatalytic reaction did not occur at a temperature lower than 30 °C. The spectra still remained at 309 nm with 30 min irradiation. The new band appeared when the temperature was increased to 50 °C and its intensity increased with temperature until 80 °C (Fig. 4). Absorbance decreased over 80 °C due to the decomposition of flunitrazepam.

The optimum conditions of irradiated sample were 8 mg/ml of ZnO at 80 °C for 30 min which were tested with the Rohypnol solution. The tendency of the photocatalysis reactions was coincident among those from flunitrazepam and Rohypnol. The wavelengths and the absorbances are similar but the absorption baselines of flunitrazepam and Rohypnol are difference leading to unequal integrated areas (Fig. 5). It can be concluded that the photocatalytic reaction by ZnO can be applied to detect Rohypnol in coloured spirits. The thermal degradation did not affect the detection.

Table 1 Optical properties of flunitrazepam and Rohypnol in the mixture solution of ethanol and soda water.

Sample	$\lambda_{\max}$, nm (ε, $M^{-1}cm^{-1}$)	
	Flunitrazepam	Rohypnol
Before irradiation	253 (17 231)	253 (18 400)
	309 (10 825)	304 (13 169)
After irradiation with ZnO	254 (15 094)	253 (17 164)
	345 (11 813)	345 (11 088)

According to these results, the ZnO particles were added into the sample containing both coloured spirit and flunitrazepam in order to remove the colour from the spirit and to change the absorbing species of flunitrazepam. After the solution was irradiated under 3×15 W blacklight fluorescence lamps for 30 min at 80 °C, a new absorption band was found at 345 nm and the absorbance of spirit was decreased for 20% (Fig. 6). The optical properties of flunitrazepam and Rohypnol before and after irradiation are collected in Table 1.

The suitable parameters like reaction temperature, irradiation time, and amount of ZnO concentration will be discussed later. In order to identify the new species after irradiation, the illuminated solution

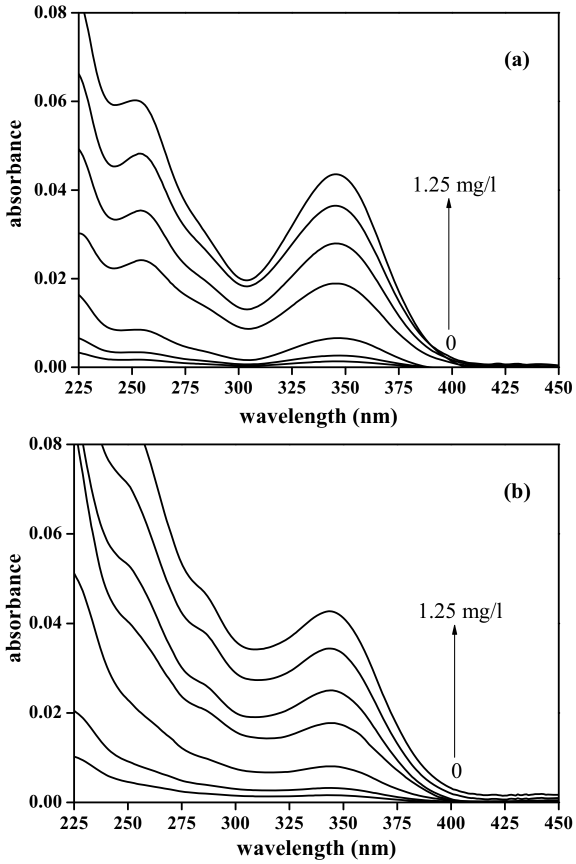


Fig. 5 Absorption spectra of (a) various concentrations of standard flunitrazepam in ethanol and soda water (b) various concentrations of Rohypnol in ethanol and soda water at the optimum condition: ZnO = 8 mg/ml, reaction temperature = 80 °C, and irradiation time = 30 min.

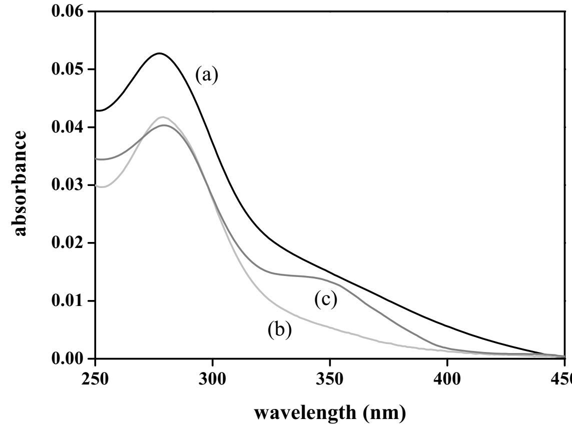
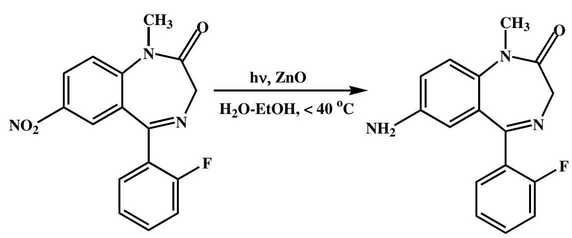


Fig. 6 Absorption spectra of (a) colour spirit before irradiation, (b) colour spirit after irradiation, and (c) flunitrazepam in colour spirit solutions after irradiation at the condition: ZnO concentration = 8 mg/ml, reaction temperature = 80 °C, and irradiation time = 30 min.



Flunitrazepam **7-amino flunitrazepam**

Fig. 7 Photocatalytic reduction of flunitrazepam.

was introduced into a mass spectrometer. The most important ions, based on m/z ratio were: 283, 69, 255, 238, 178, and 149 while the mass spectrum of flunitrazepam showed the m/z: 312, 313, 286, 266, 238. There was an important peak at $m/z = 283$ which is the characteristic peak of 7-amino-flunitrazepam and its fragmentation pattern[1]. The reduction of nitro- to the amino- compounds in an alcoholic solution can occur using metal oxide photocatalysts[24]. In general, the α-hydroxyalkyl radical is generated by photocatalytic reaction of semiconductor in alcoholic solution. It can react with nitro organic directly. Its radical is an active reducing agent[2,3]. The proposed mechanism is shown in Fig. 7 and equations (1)–(5):

$$\mathrm{ZnO} + h\nu \longrightarrow \mathrm{e}^-_{\mathrm{cb}} + \mathrm{h}^+_{\mathrm{vb}} \tag{1}$$

$$\mathrm{H_2O} + \mathrm{h}^+_{\mathrm{vb}} \longrightarrow {}^{\cdot}\mathrm{OH} + \mathrm{H}^+ \tag{2}$$

$$\mathrm{O_2} + \mathrm{e}^-_{\mathrm{cb}} \longrightarrow \mathrm{O_2}^{-\cdot} \tag{3}$$

$${}^{\cdot}\mathrm{OH} + \text{spirit pigment} \longrightarrow \text{oxidized products} \tag{4}$$

$$\mathrm{O_2}^{-\cdot} + \text{flunitrazepam} \longrightarrow \text{7-amino-flunitrazepam} \tag{5}$$

The ZnO catalyst is exposed to UV light in the UV reactor, electrons are promoted from the valence band to the conduction band. A pair of electron ($\mathrm{e}^-_{\mathrm{cb}}$) and hole ($\mathrm{h}^+_{\mathrm{vb}}$) are generated in a conduction band and valence band, respectively. The hole moves to the surface of the ZnO then reacts with H_2O in soda water producing hydroxyl radical (${}^{\cdot}$OH) while the electron reacts with O_2 producing superoxide anionic radical ($\mathrm{O_2}^{-\cdot}$). The ${}^{\cdot}$OH can react and discolouration pigments appear in the spirit. The oxidized form is generated and then decreases the spirit absorbance as shown in Fig. 6a and Fig. 6b whereas the $\mathrm{O_2}^{-\cdot}$ radical can reduce flunitrazepam to be 7-amino-flunitrazepam. A new band can be readily observed at 345 nm (Fig. 6c). There has been a report of the photochemical reduction of flunitrazepam in the presence of methanol and irradiated at 300 nm[25]. The 7-amino flunitrazepam product is obtained. In our experiment, the absence of ZnO photocatalyst in alcohol under blacklight fluorescence lamps, a costless and commercially available item, cannot stimulate the flunitrazepam reduction.

Calibration and detection limit

Calibration curves for preliminary quantitative analysis of flunitrazepam and Rohypnol detections were plotted with 7 different concentrations ranging from 0.05–1.25 mg/l (not shown). The plots of integrated areas of 345 nm absorption bands after irradiation with ZnO of standard flunitrazepam and Rohypnol are both linear ($R^2 = 0.9976$ and 0.9925, respectively). The extinction coefficients of standard flunitrazepam and Rohypnol are $3.94 \times 10^5\ M^{-1}$ and $2.26 \times 10^5\ M^{-1}$, respectively. The detection limit was determined from the 0.25 mg/l of flunitrazepam.

According to Miller and Miller[26], the limit of detection and limit of quantification based on S/N of 3 and 10 of standard flunitrazepam are 0.024 and 0.082 mg/l, respectively, which are lower than that obtained from Rohypnol experiment (0.092 and 0.306 mg/l).

Coloured spirits

Distributed three colour spirit brands in Thailand were selected to test the analytical method in the real samples. The flunitrazepam 0.25 mg/l was spiked to the coloured spirit solutions in order to check the detection of flunitrazepam. With this amount of flunitrazepam, it is able to observe the absorbance signal obviously under Lambert and Beer's law. The absorption spectra of the absence and presence of 0.25 mg/l flunitrazepam in coloured spirit after irradiation with ZnO particles were compared at the optimum condition. After irradiation, the new band at $\lambda_{\max} \sim$ 345 nm can be observed (Fig. 8). The minimum concentration of Rohypnol that can be detected with the photocatalytic process is 0.10 mg/l.

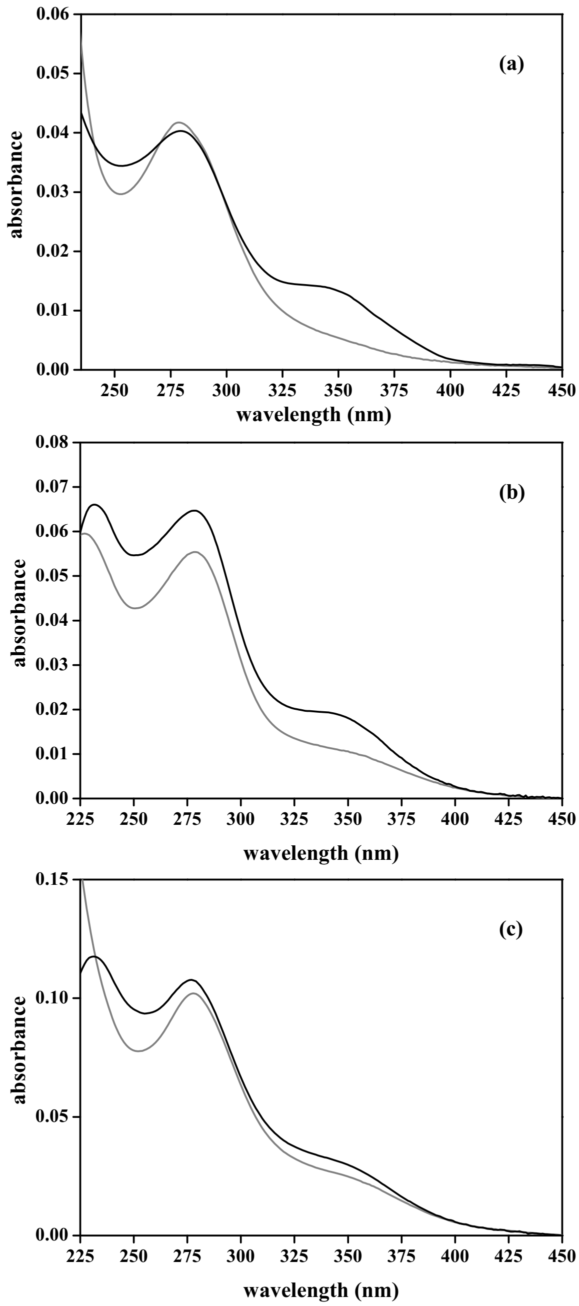


Fig. 8 Absorption spectra of irradiated colour spirit (grey line) and flunitrazepam added colour spirit (black line) from three colour spirit brands in Thailand; (a) Master blend, (b) 100 Pipers, and (c) Regency.

CONCLUSIONS

The photocatalytic process of flunitrazepam and Rohypnol by ZnO was valid. The 7-amino reduction species occurred in the solution of alcoholic and aqueous solution. The MS results demonstrated that there were changes at the m/z characteristic peak. The 7-amino-flunitrazepam with $m/z = 283$ and its pattern, the characteristic species occurs after irradiation. The new absorption band at maximum wavelength of 345 nm was observed. The rates of photocatalytic reaction were affected by the reaction temperature, ZnO concentration, and irradiation time. The best conditions were found at 8 mg/ml of ZnO while the reaction temperature was 80 °C with the irradiation time of 30 min. This method can be applied to detect the standard flunitrazepam and Rohypnol tablet in coloured spirit. The detection limits of Rohypnol in coloured spirit was 0.10 mg/l. The ZnO catalyst was proposed to discolour the spirit's colour and reduce the flunitrazepam to the 7-amino-flunitrazepam.

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