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# Separation and structural elucidation of a novel analogue of vardenafil included as an adulterant in a dietary supplement by liquid chromatography–electrospray ionization mass spectrometry, infrared spectroscopy and nuclear magnetic resonance spectroscopy

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ABSTRACT

MEGATON, a dietary supplement, was analyzed in order to detect PDE-5 inhibitors and their analogues. A new analogue of vardenafil could be detected by high-performance liquid chromatography (HPLC) analysis with a photodiode array detector (PDA). This compound was compared with sildenafil, tadalafil, and vardenafil as well as their structurally modified analogues such as hongdenafil and homosildenafil. The structure of this compound was elucidated by mass spectrometry (MS), infrared (IR) spectroscopy and one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy. When compared with vardenafil to verify the structural difference, this compound had an acetyl group instead of a sulfonyl group in the pyrazolopyrimidine portion without any substitution in the piperazine ring of the molecule. This compound was identified as 2-(2-ethoxy-5-(2-(4-ethylpiperazin-1-yl)acetyl)phenyl)-5-methyl-7-propyl-imidazo(5,1-f)-(1,2,4)triazin-4(3H)-one, which is also called acetylvardenafil.


## 1. Introduction

Dietary supplements should fulfill high expectations in food products designed to meet the consumer's desire for a healthy lifestyle. These products relieve hunger and supply additional nutrients to humans. These products also prevent consumers from nutrition deficiency and increase the physical and mental health of consumers. The global market of healthy food is expected to greatly expand in the future. However, the ethics of the market has not risen in accordance with the economic growth. In recent years, there have been several reports about the adulteration of herbal dietary supplements with one of the three synthetic erectile dysfunction (ED) drugs which may cause a serious threat to human health [1–7]. Dietary supplements with these ED drugs can be got with ease because they are easy to purchase without needing a prescription and there are many places to buy dietary supplements owing to an increasing need for health.

Sildenafil citrate is the first drug approved for the treatment of erectile dysfunction (ED), but it was initially developed for the purpose of treating angina pectoris. After it was discovered to be effective in anti-impotence during clinical trials, it was introduced into the market in 1998. Today, there are three anti-impotence drugs that have been approved by the Food and Drug Administration (FDA) for the treatment of ED: sildenafil citrate (Viagra®, manufactured by Pfizer), vardenafil hydrochloride (Levitra®, manufactured by Bayer) and tadalafil (Cialis®, manufactured by Lilly). With the commercial success of these drugs, many herbal products have also been marketed as natural alternatives to these synthetic prescription drugs [8].

A dietary supplement, known as MEGATON, was imported to Incheon airport in South Korea from the USA in capsule forms. It was tested for the presence of sildenafil, tadalafil, and vardenafil as well as their structurally modified analogues, using the HPLC method. The experimental procedure was followed by an official method for detecting hazardous substances in foods [9]. The structures of sildenafil and vardenafil show only minor differences, while tadalafil differs markedly in its chemical structure [10–14]. In the current study, a novel analogue of vardenafil could be identified. The structure of the compound was elucidated as

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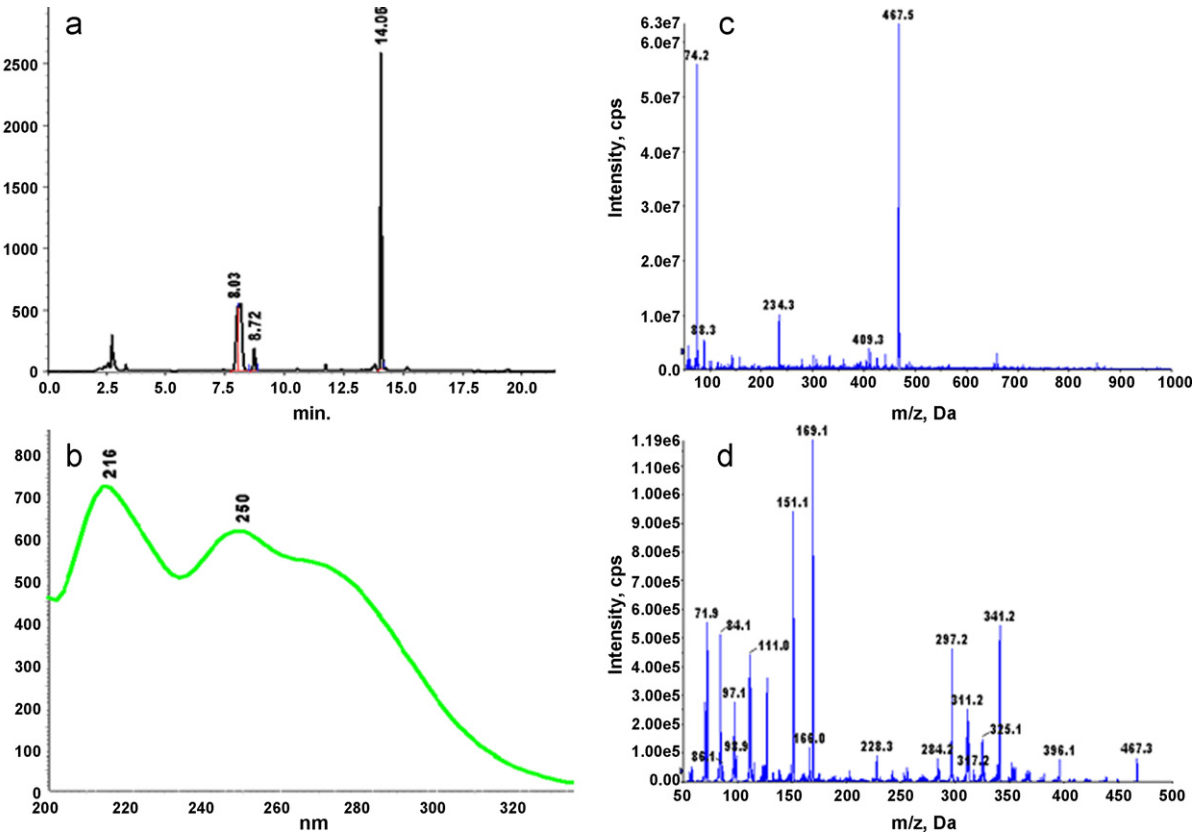


**Fig. 1.** (a) HPLC chromatogram of MEGATON, (b) UV spectrum of a HPLC peak at 14.06 min, (c) mass spectrum of the compound at 14.06 min, and (d) product ion spectrum of the 467 *m*/*z* mass peak.

**Table 1**
$^{1}$H-nuclear magnetic resonance (NMR) and $^{13}$C-NMR spectral data for the unknown product in $CDCl_3$ and vardenafil in DMSO-$d_6$.

| Number | $\delta$ ($^{13}$C) ($CDCl_3$) | $\delta$ ($^{1}$H) ($CDCl_3$) | $\delta$ ($^{13}$C) vardenafil (DMSO-$d_6$) | $\delta$ ($^{1}$H) vardenafil (7) (DMSO-$d_6$) |
|---|---|---|---|---|
| 1 | 146.3 | | 144.4 | |
| 3 | 140.1 | | 137.6 | |
| 4 | 155.3 | | 155.0 | |
| 5 | – | 10.12 (1H, br, s) | – | 11.82 (1H, br, s) |
| 6 | 145.6 | | 146.1 | |
| 9 | 113.8 | | 113.3 | |
| 10 | 14.7 | 2.62 (3H, s) | 14.1 | 2.50 (3H, s) |
| 11 | 28.1 | 3.01 (2H, t, $J$ = 7.6) | 27.1 | 2.86 (2H, t, $J$ = 7.4) |
| 12 | 21.1 | 1.89 (2H, m) | 20.2 | 1.76 (2H, m) |
| 13 | 14.1 | 1.04 (3H, t, $J$ = 7.4) | 13.7 | 0.94 (3H, t, $J$ = 7.4) |
| 14 | 118.3 | | 126.4 | |
| 15 | 131.5 | 8.74 (H, d, $J$ = 2.3) | 130.3 | 7.97 (H, d, $J$ = 2.4) |
| 16 | 129.7 | | 120.9 | |
| 17 | 133.4 | 8.21 (H, dd, $J$ = 8.8, 2.3) | 132.2 | 7.95 (H, dd) |
| 18 | 112.6 | 7.11 (H, d, $J$ = 8.8) | 113.6 | 7.45 (H, d, $J$ = 8.8) |
| 19 | 160.5 | | 160.7 | |
| 20 | 65.7 | 4.32 (2H, q, $J$ = 7.0) | 65.1 | 4.24 (2H, q, $J$ = 6.9) |
| 21 | 14.6 | 1.56 (3H, t, $J$ = 7.0) | 14.3 | 1.35 (3H, t, $J$ = 6.9) |
| 22 | 194.7 | | – | – |
| 23 | 64.8 | 3.78 (2H, s) | – | – |
| 25,29 | 53.6 | Broad band just above 2.62 (4H, br) | 43.0 | 3.50,3.81 (4H, br) |
| 26,28 | 52.7 | 2.45 (4H, br) | 49.4 | 3.81 (4H, br) |
| 30 | 52.4 | 2.44 (2H, q, $J$ = 7.2) | 50.6 | 2.90 (2H, q) |
| 31 | 12.0 | 1.09 (3H, t, $J$ = 7.2) | 8.7 | 1.23 (3H, t, $J$ = 7.2) |

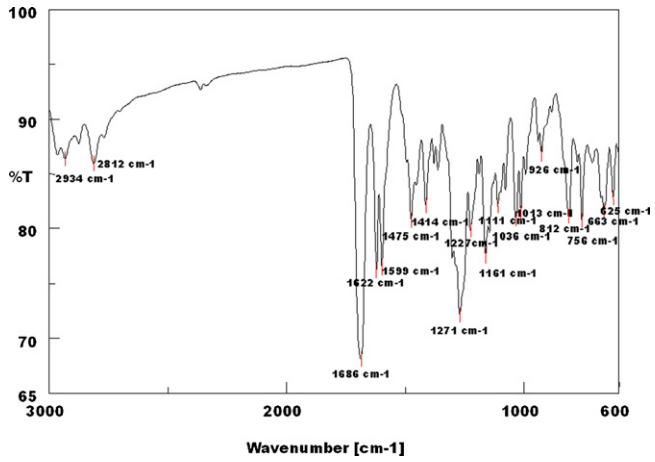


**Fig. 2.** IR spectrum of the fraction of the unknown compound.

2-(2-ethoxy-5-(2-(4-ethylpiperazin-1-yl)acetyl)phenyl)-5-methyl -7-propyl-imidazo(5,1-f)-(1,2,4)triazin-4(3H)-one, in which a sulfonyl group of vardenafil was substituted by an acetyl group.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Vardenafil hydrochloride, hongdenafil (acetildenafil) citrate were sourced from the Korea Food and Drug Administration (Seoul, Korea). 18 MΩ purity grade water was prepared from Milli-Q Water System (Millipore, Bedford, MA, USA). Formic acid and acetic acid (reagent grade, min. 98%), acetonitrile (HPLC-grade), dichloromethane and all other reagents were purchased from Sigma (St. Louis, MO, USA).

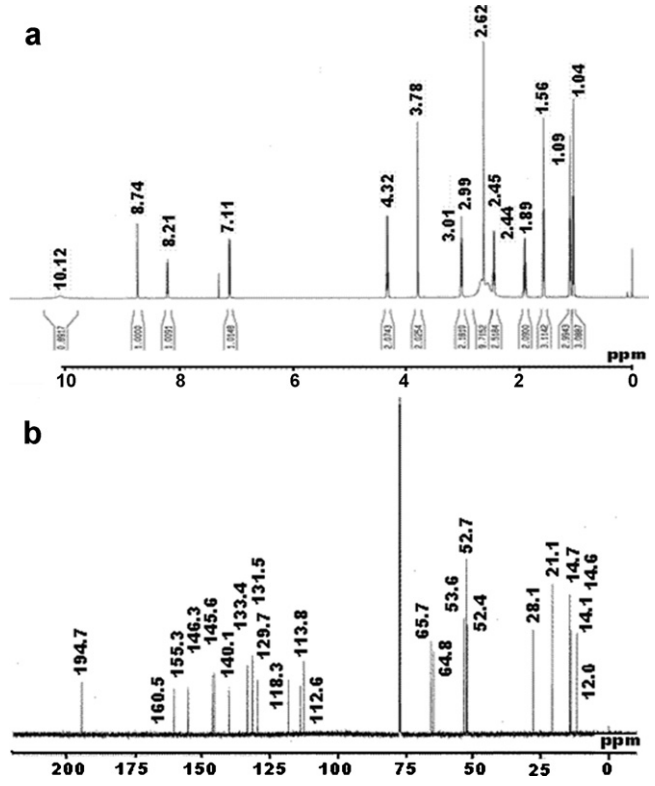


**Fig. 3.** (a) $^{1}$H-NMR, and (b) $^{13}$C NMR spectra of the unknown compound.

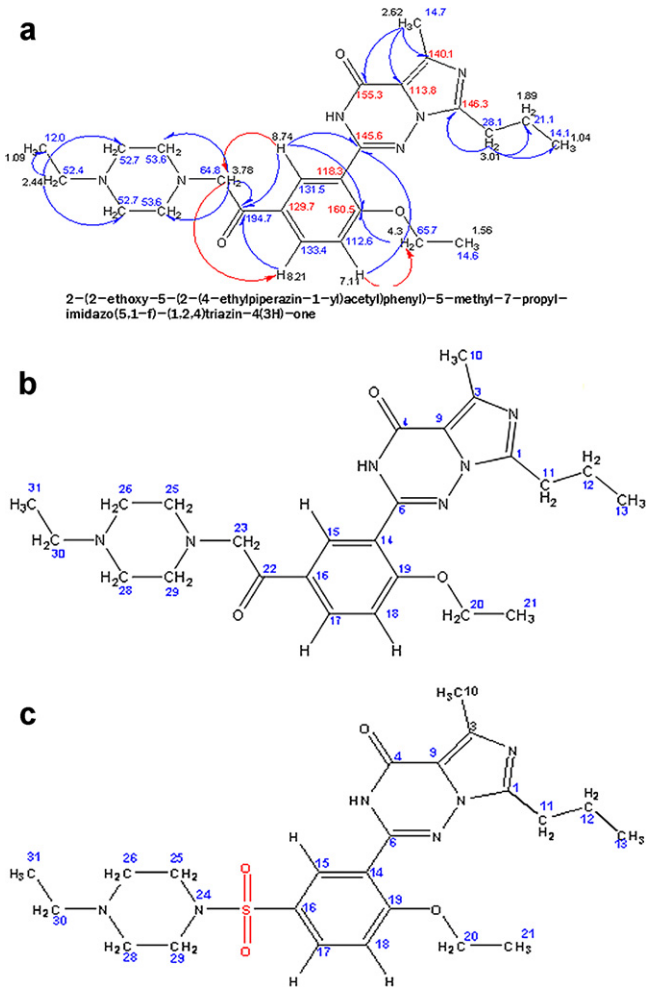


**Fig. 4.** Proposed structure of (a) the unknown compound from the results of correlation spectroscopy (COSY), nuclear overhauser effect spectroscopy (NOESY) and heteronuclear multiple bond correlation (HMBC) data in $CDCl_3$, (b) expected structure of the unknown compound, and (c) vardenafil structure: arrows mean correlations from proton(s) stated to the indicated carbon(s).

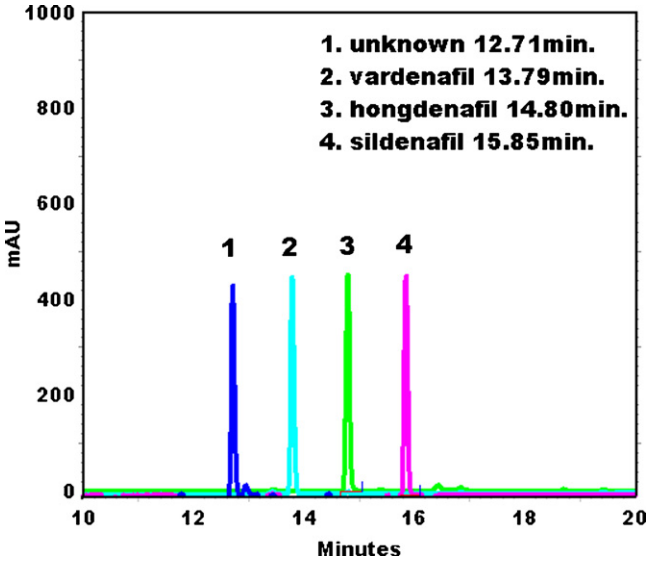


**Fig. 5.** HPLC chromatogram of 1) unknown, 2) vardenafil, 3) hongdenafil, and 4) sildenafil.

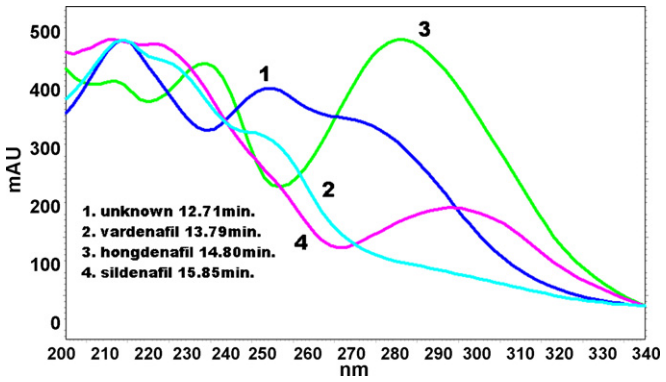


**Fig. 6.** HPLC/UV spectrum of unknown, vardenafil, hongdenafil and sildenafil.

### 2.2. Sample and extraction

A dietary supplement, known as MEGATON, includes nutritional yeast with brewer's yeast (65%), vitamin C (15%) and dried Ganoderma lucidum Krast powder (8.25%). The packing claimed that the product contained naturally occurring B vitamins that are essential for carbohydrate metabolism and energy production, minerals and amino acids. The contents from the dietary supplement containing brewer's yeast were mixed and homogenized. For the preliminary screening, a total of 1 g of the contents was extracted with 50 mL of methanol/water (70/30) solution and filtered through a 0.45 μm poly vinylidene fluoride (PVDF) filter. The filtered solution was directly injected into the HPLC system. If it was necessary, the solution was diluted by 10 times or 100 times with methanol/water (70/30) solution before HPLC analysis.

A large scale approach was also taken to perform the fractionation of unknown components with a semi-Prep. LC system. An amount of 12.593 g of the homogenized brewer's yeast was extracted two times with 75 mL of methanol/water (70/30) solution. The pH of the solution was adjusted to about 9 or 10 with 1% sodium bicarbonate and sodium carbonate and the solution was extracted with 75 mL of dichloromethane three times. The dichloromethane layer was evaporated and concentrated to dryness at 35 °C. The solid extract was re-dissolved in 100 mL of 50% methanol and re-adjusted to pH 1 or 2 with dilute hydrochloric acid. The impurities in the solution were removed with 75 mL dichloromethane two times and the methanol layer was adjusted to above pH 9 with 1% sodium bicarbonate and sodium carbonate and extracted with 75 mL of dichloromethane two times. The dichloromethane solution was evaporated and concentrated to dryness at 35 °C again. The solid residue was redissolved and injected into the semi Prep. system to isolate and purify the unknown compound of interest. As the extraction progressed, each solution was analyzed with the HPLC system to estimate a purity of the unknown interesting component.

### 2.3. HPLC and semi-Prep. LC analytical condition to isolate unknown compound

A Shiseido Nanospace SI-2 HPLC system with a PDA detector was used and controlled by EZChrom Elite Client/Server software. A Shiseido CapcellPAK C18 MG II (4.6 mm i.d. × 250 mm, 5 μm) column was used in the HPLC analysis for retention and separation of the analytes. The working standard solutions and sample solutions (inj. vol. = 10 μl) were analyzed using a 40 °C column oven with a flow rate of 1.0 mL/min. An aqueous solution with 0.5 mM sodium 1-hexanesulfonate and 0.1% phosphoric acid (A) and 95% acetonitrile (B) were used as a mobile phase for the HPLC analysis. The gradient program consisted of a linear step from 5% for 5 min to 100% of solvent B in 25 min. A PDA spectrum was obtained from 200 to 340 nm and HPLC chromatogram peaks were detected at 210 and 290 nm using dual channels. For the isolation and purification of the unknown compound, the chromatographic system consisted of a Shiseido Nanospace SI-2 pump coupled with a Rheodyne manual injector, a Shiseido Nanospace SI-2 UV detector, and a Donam dsChrom workstation program. The separation was performed on a Gemini C18 (10 mm i.d. × 250 mm, 5 μm) column and investigated at 290 nm.

Further separation was performed with a semi-Prep. LC system using 20% acetonitrile in 0.1% acetic acid with a flow rate of 3 mL/min. Fractions containing unknown peaks were collected manually after 200 μL injection into the semi-Prep. LC system until sufficient amounts for the instrumental analysis were collected. Finally, the fractions acquired from the semi-Prep. LC was washed with 75 mL of dichloromethane to purify, and the dichloromethane layers were evaporated. This purified compound was analyzed by using MS, IR, $^{1}$H NMR and $^{13}$C NMR spectra.

### 2.4. LC–MS sample and standard preparation

Stock solutions of standards were each prepared with a concentration of approximately 200 μg/mL in methanol and stored in the refrigerator. Working standards (0.1–0.5 μg/mL) were freshly prepared for every experiment by diluting stock standards with acetonitrile. A portion of the extract in the 2.1 sample preparation was filtered through a 0.2 μm nylon syringe filter and diluted further with acetonitrile prior to analysis. The use of acetonitrile as a diluent and extraction solvent was necessary due to the poor solubility of tadalafil in methanol [10].

### 2.5. LC–MS instrument parameters

In the LC–MS experiment, the chromatographic column was a C18 column and the mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as recommended [7,10,16,17]. The control of the system and data acquisition was performed by Analyst software Ver. 1.4.1. The running time for the total analysis was 15 min, including a column equilibration step between samples. An Agilent 1200 HPLC system linked with an API 4000 Q mass spectrometer (Applied Biosystem, Foster City, CA, USA) was used with electrospray ionization in the positive ion mode. The operating MS parameters were optimized as follows: curtain gas, 10 psi; ion spray voltage,+5500 V; collision gas, nitrogen; resolution of Q1 and Q3, unit; ion source gas 1, 10 (arbitrary unit); declustering potential, 70 V; and entrance potential, 10 V. To acquire the mass spectrum of the unknown compound, the profile scan mode was operated. The profile scan was performed with Q1 scanning from 50 to 1000 amu for 3 s with a step size of 0.10 amu. Data acquisition for the product ion spectrum was performed by working in product ion scan mode. The MS parameters were as follows: collision energy, 55 V; MS full scan range, $m/z$ 50–500; step size, 0.10 amu; and scan time, 4.0 s. The operating parameters were optimized as follows: curtain gas, 10 psi; ion spray voltage, +5500 V; collision gas, nitrogen; and resolution of Q1 and Q3, unit. A CapcellPak C18 MG II column with a 5 μm-particle size (150 mm × 2.1 mm) (Agilent Technologies, Wilmington, DE, USA) was used and operated at 35 °C. The two mobile solvents consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The initial mobile phase consisted of 5% B and was changed linearly to 95% B over 0–8 min, maintained at 95% B for 3 min, then returned to 5% B in 1 min. The system was pumped at a flow rate of 250 μL/min.

### 2.6. NMR and IR

For NMR analysis, a Bruker AVANCE 600 spectrometer (14.1 T, Bruker BioSpin GmbH, Rheinstetten, Germany) with a 5 mm probe was used. $^1$H NMR and $^{13}$C NMR spectra were acquired after dissolving 20 mg of the fraction in 0.75 mL of deuterated chloroform ($CDCl_3$) as a solvent. A Bruker IFS-66 spectrometer (Bruker Optik GmbH, Ettlingen, Germany) scanned the sample disk prepared after scattering 1 mg of the fraction onto 50 mg of potassium bromide for the IR analysis. The scanned region was in the wave number range of 400–4000 $cm^{-1}$, and the wave number range of 600–3000 $cm^{-1}$ was shown in the results.

## 3. Results and discussion

For the HPLC chromatogram analysis of the dietary supplement, a single large unknown peak appeared at 14.06 min (Fig. 1a). The peak shows maximum intensity at 216 nm and 250 nm by HPLC/PDA analysis (Fig. 1b). The protonated molecular mass of this unknown compound was found to be *m*/*z* 467.5, identified by direct flow injection electrospray ionization (ESI) scan mass analysis (Fig. 1c). The *m*/*z* 467.5 ion was analyzed by ESI tandem product ion scan analysis resulting from collision induced dissociation of the protonated ion (Fig. 1d). The *m*/*z* values of 151.1, 169.1, 284.2, 297.2, 311.2 325.1, 341.2, 396.1 and 467.3 were shown in the product mass spectrum of the ion which had an *m*/*z* of 467.5. The three ions with *m*/*z* values of 151.1, 169.1 and 284.2 among these ions would have originated from a vardenafil hydrolysis product produced from the cleavage at the nitrogen–sulphur bond, followed by the subsequent loss of the $SO_3$ molecule from the sulphonic acid compound [8,10,11,14,15]. The four ions with *m*/*z* values of 467.3, 396.1, 341.2 and 297.2 were proposed to have been fragments across the piperazine ring [16,17]. It has been reported by Reepmeyer and Woodruff [15,17] that the ions with *m*/*z* 151, 169 and 284 are caused by the cleavage of vardenafil at the nitrogen–sulphur bond, followed by the subsequent loss of the $SO_3$ molecule from the sulphonic acid compound. The IR spectrum showed absorption bands at 1622 $cm^{-1}$ and 1475 $cm^{-1}$ for an aromatic ring (C=C), 1686 $cm^{-1}$ for an unsaturated carbonyl group (C=O), and 1271 $cm^{-1}$ and 1036 $cm^{-1}$ for a carbon–oxygen vibration mode (C–O) (Fig. 2). The $^1$H NMR and $^{13}$C-NMR spectral data of the unknown compound in the herbal product are compared to the vardenafil data in Table 1 [14]. The $^1$H NMR spectra of the unknown compound in the herbal product were very similar to those of vardenafil (Fig. 3, Table 1). The chemical shift differences in the $^{13}$C-NMR spectra between vardenafil and the unknown compound were not larger than 1 ppm, except for carbons at $\sigma$131.5 (C-15), 133.4 (C-17), 53.6 (C-25, 29) 52.7 (C-26, 28), 52.4 (C-30) and 12.0 (C-31) (Fig. 4). However, only one unassigned peak for the unknown compound at $\sigma$3.78 (C-28, 2H, s) in the $^1$H NMR spectral data (Fig. 3a, Table 1) was absent in the NMR spectrum of vardenafil. In addition, two unassigned peaks $\sigma$194.7 and $\sigma$64.8 were not shown in the $^{13}$C NMR spectrum of vardenafil (Fig. 3b). Comparing two $^1$H- and $^{13}$C-NMR spectra of the unknown compound with those of vardenafil, a structure modification of the sulfonyl group could be suggested. In the $^1$H–$^1$H correlation spectroscopy (COSY), H-15 and H-23 were correlated with H-23 and H-17, respectively (Fig. 4a,b). These results were further confirmed by a heteronuclear multiple bond correlation (HMBC) analysis. There are several reports [16–20] on the characterization of sildenafil and its analogues, such as hongdenafil (acetildenafil). The hongdenafil was elucidated as the substitution of the sulfonyl group [16,17,19] to the methyl carbonyl group (–$CH_2$C=O–) of sildenafil. Based on the UV spectra, the mass spectral fragmentation patterns and the $^1$H NMR spectra of the unknown compound in the dietary product, we concluded that vardenafil and the unknown compound have the same structure containing a minor difference. The unknown compound in the dietary product is an analogue of vardenafil, of which systematic name is 2-(2-ethoxy-5-(2-(4-ethylpiperazin-1-yl)acetyl)phenyl)-5-methyl-7-propyl-imidazo(5,1-f)-(1,2,4)triazin-4(3H)-one and its protonated molecular mass *m*/*z* is 467.5, which is commonly referred to as acetylvardenafil [21]. The structures of vardenafil and the new analogue are shown in Fig. 4. Therefore, a difference in polarity can directly affect retention times in the HPLC chromatogram for acetylvardenafil, vardenafil, hongdenafil and sildenafil (Fig. 5). The retention time of acetylvardenafil was observed to be lower than that of the vardenafil. Similar results were also obtained between hongdenafil and sildenafil. Namely, hongdenafil showed faster mobility in the C18 reverse column, compared with that of sildenafil. Also, the UV peak at 220–240 nm of the unknown compound disappeared or shifted in the HPLC/UV spectrum, compared with those of vardenafil and sildenafil (Fig. 6). This spectral change was mainly contributed by the alteration of the functional group, that is, the change from a sulfonyl group by an acetyl group.

## 4. Conclusions

A brewer's yeast, marketed under the name "MEGATON", was found to contain a synthetic analogue of vardenafil, in which a sulfonyl group was changed by an acetyl group. The structure of this compound was analyzed and confirmed as a substituted product of vardenafil through analysis utilizing HPLC-PDA, LC/MS/MS, $^1$H- and $^{13}$C-NMR.

Such analogues are difficult to identify by ordinary methods because of their similarities in structures. They might be intentionally used as an attempt to evade regulatory inspection. Our findings showed that surveillance of over-the-counter health products is necessary and should be extended to cover registered pharmaceutical compounds as well as their illicit analogues.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2010.09.022.

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