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2D and 3D DOSY ^{1}H NMR, a useful tool for analysis of complex mixtures: Application to herbal drugs or dietary supplements for erectile dysfunction

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ABSTRACT

Seventeen herbal dietary supplements, marketed as natural substances for the enhancement of sexual function, were analyzed by diffusion ordered spectroscopy (DOSY) ^{1}H NMR. The method allowed a global analysis of the samples with detection of both active and inactive ingredients present in these complex matrixes. Eight formulations contained compounds related to the synthetic phosphodiesterase-5 inhibitors. Sildenafil, tadalafil, vardenafil, hydroxyhomosildenafil, thiosildenafil, and the newly identified adulterant thiomethisosildenafil were detected. Quantification of these active ingredients was carried out by HPLC or NMR. In addition to these actives, about 30 compounds or excipients were characterized. This study ended up with a three-dimensional DOSY–COSY ^{1}H NMR experiment on a herbal formulation which provided both virtual separation and structural information.

1. Introduction

Over the last few decades there has been an exponential growth in the field of herbal medicine. It is getting popularized in developing and developed countries and plays an active part in people's health care. These products are regarded by many as being harmless because of their natural origin, and helpful to the treatment of some chronic diseases and to the maintenance of physical fitness.

Nevertheless some manufacturers have included synthetic drugs in the formulation process of their products marketed as "herbal medicines" or "dietary supplements", in order to improve the effects of their products. In recent years, in particular, there have been several reports about herbal drugs marketed for sexual dysfunction which had been adulterated with synthetic phosphodiesterase-5 (PDE-5) inhibitors (sildenafil, tadalafil and vardenafil) or analogues in which minor modifications had been brought to the molecular structure of PDE-5 inhibitor drugs [1–20]. The adulteration of "natural" herbal products with erectile dysfunction drugs or analogues is a growing trend and poses a health threat to patients who unwittingly consume a synthetic drug that has been untested for safety.

Reported methods concerning screening for illegal adulterations in herbal drugs are mainly HPLC, LC/MS and LC/MS/MS [4,7,10]. NMR has been carried out in a few studies for structural identification on isolated compounds [2,3,8,9,13,15,18,20]. However, herbal products often contain a mixture of herbs and other natural products. The compositions of those products are considerably variable among brands and chromatographic methods are sometimes too selective and do not supply any global information.

In the present study we focused on an application of 2D diffusion ordered spectroscopy ^{1}H nuclear magnetic resonance (2D DOSY ^{1}H NMR) for accurate identification of herbal drug composition. The main advantage of DOSY NMR is to provide global information. Moreover, the method is non-selective and requires no prior knowledge of the structures of the various components present in the mixture. The method has been previously used for the analysis of generic and counterfeit drugs [21–23].

To the best of our knowledge, it is the first time thiomethisosildenafil is described in literature as an analogue of sildenafil and as an adulterant in herbal products. This study is also the first application of a 3D DOSY–COSY experiment to an herbal mixture.

2. Experimental

2.1. Commercial herbal drugs

Seventeen commercial formulations of herbal drugs or dietary supplements marketed for sexual dysfunction were analyzed.

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Table 1
Herbal commercial formulations analyzed in this study.

	Formulation name	Batch number	Expiry date	Manufacturer name	Country of manufacturing	Product form	Color
1	Masculin 1	20060218	02/2009	Kangli-Qinghai	China	Tablet	Brown
2	Yeilus Miracule	020-61131315-315	04/2009	Hongkong Tianlong	China	Capsule	Red
3	Sex Gold	Q/WSJQ-2005	12/2008	Yikanda	China	Capsule	Yellow and black
4	Chui Hua San	20014423	11/2006	Junen Company	Taiwan	Capsule	Yellow
5	Penis volume plus	z/m p 002-2002	12/2007	Paramedical Products of Beijing	China	Capsule	Red and black
6	Viager (for female)	SZ/O4BJ361-2004	10/2009	Shanghai Ruizhi Health Food Co. Ltd.	China	Capsule	Pink
7	Herbal V	TLI6001	06/2009	Unknown	India	Capsule	Red
8	Vigueur-7	P060297	04/2010	Laboratoire Lebeau	France	Capsule	Transparent
9	Penis Kraft	106	03/2009	Milan Arzneimittel	Germany	Tablet	Orange
10	ViaMax sensitive desire	18992006	07/2009	Bionordica Cosmetics and Supplements	Sweden	Capsule	Red
11	Power Tab	23542007	08/2010	NordMax Team Ltd.	Estonia	Capsule	Cyan
12	Active man herb	0025	03/2009	Ibnalnafis Herbs	Syria	Powder	
13	Active women herb	0025	03/2009	Ibnalnafis Herbs	Syria	Powder	
14	Stamina-Rx	0614	07/2008	Hi-Tech Pharmaceuticals	USA	Tablet	Blue
15	Max Size	612086	12/2009	M.D. Science Lab	USA	Tablet	Brown
16	Herbal Viva	605132	05/2009	M.D. Science Lab	USA	Capsule	Transparent
17	Start Up	–	10/2010	Lab Start Up	Spain	Capsule	Blue and white

Formulations **12**, **13** were bought in Syria, **2**, **4**, **5**, **6** in China, and **17** in France. Other formulations were purchased on the internet. The list of the analyzed tablets or capsules is given in Table 1. All samples, as received, were stored in the dark at ambient temperature and humidity. They were all analyzed before expiry dates.

2.2. Sample preparations

2.2.1. NMR analysis

The tablet was powdered (or capsule emptied) and dissolved in 5 mL of solvent under magnetic stirring for 30 min, then sonicated for 10 min. The suspension was centrifuged (10 min, 3000 rpm) and the supernatant analyzed. Two solvents were used D_2O and CD_3CN:D_2O (80:20, v/v). For quantitative analysis of PDE-5 drugs or analogues found in some formulations, the tablets were powdered (or capsules emptied) and dissolved in 100 mL of methanol under magnetic stirring for 45 min, then sonicated for 10 min. An aliquot of 2 mL was evaporated to dryness and the residue dissolved in 2 mL of a 2×10^{-3} mol L^{-1} solution of TSP (trimethylsilyl-2,2,3,3-tetradeuteropropionic acid, sodium salt) in MeOH-d_4 before the NMR analysis. The experiment was made in triplicate.

2.2.2. Chromatographic analysis

For all chromatographic analyses, the tablets were powdered (or capsules emptied) and dissolved in 100 mL of methanol under magnetic stirring for 2 h, then sonicated for 30 min. The solutions were then diluted 10-fold for quantitative analysis by LC–DAD or 100-fold for LC–MS analysis and then filtered through a 0.45 μm filter before injection. The experiment was made in triplicate.

2.3. ^1H and DOSY ^1H NMR

The spectra were recorded on a Bruker Avance 500 spectrometer in two different solvents, heavy water (D_2O) and a mixture CD_3CN:D_2O (80:20). The recording and processing conditions for ^1H and 2D DOSY ^1H NMR spectra have already been described [22]. For 2D DOSY ^1H NMR, sequence parameters were adapted in order to have the intensity of the main signals of the spectrum in the aromatic region strongly decreased (at least divided by 50) at 95% of the full gradient strength.

^1H NMR assay of vardenafil, hydroxyhomosildenafil, thiomethisosildenafil and thiosildenafil was done using TSP as internal reference of quantification. For these quantitative experiments, relaxation delay was lengthened to 3 s and a pulse width of 3.5 μs (flip angle ≈30°) was employed. The concentrations of PDE-5 analogues detected were measured by comparing the signal areas of their aromatic protons (H_7, H_8 and H_9) with that of TSP, the area of each NMR peak being directly proportional to the number of corresponding nuclei. The areas were determined using Bruker TopSpin software. Each data is the mean of at least five integrations.

For 3D DOSY–COSY ^1H NMR acquisition, a variant of 3D DQF–COSY iDOSY [24] including water presaturation pulse was recorded on formulation **1**. In the COSY dimension, 8182 × 104 data points were used, corresponding to 0.68 s for acquisition time and 8 ms for the other dimension, with a spectral window of 12 ppm and the transmitter offset frequency located at 4.704 ppm. The sineshaped gradients for DQF selection, of 4.62 and 9.25 G cm^{-1} strength, were applied for 1 ms. Twenty-eight gradient steps were used for the diffusion dimension from 5 to 95%, where 46.25 G cm^{-1} was the maximum gradient intensity. The diffusion time was 60 ms, the gradient pulse length 6.8 ms, and the recovery delay 3 ms. Water presaturation was applied during the relaxation delay of 1 s and eight scans were used for this sample (half-day experiment). The spectrum was processed in COSY dimension with Fourier transform in magnitude mode before applying the inverse Laplace transform method (MaxEnt algorithm) to obtain the DOSY dimension. The processing parameters were 192 points along the diffusion axis and 20,000 MaxEnt iterations. The algorithm was computed only on the columns with a signal-to-noise ratio above 32. 2D COSY and DOSY experiments were acquired with DQF–COSY iDOSY parameters. These experiments were processed using NPK software [25] and analyzed with NMRnotebook package [26].

2.4. LC–DAD apparatus and chromatographic conditions

HPLC was carried out using a Waters 2695 Alliance model with a Waters 2996 diode array detector. The analytical column was a reversed-phase column Luna C18 (100 × 3 mm ID; 3 μm particle size; Phenomenex, UK). The column temperature was 30 °C. The flow rate was 0.6 mL min^{-1}. A detection wavelength of 225 nm was chosen as it allows the detection of all tadalafil or sildenafil analogues.

For quantification of tadalafil content, the mobile phase consisted of a mixture (35:65, v/v) of acetonitrile and phosphate buffer

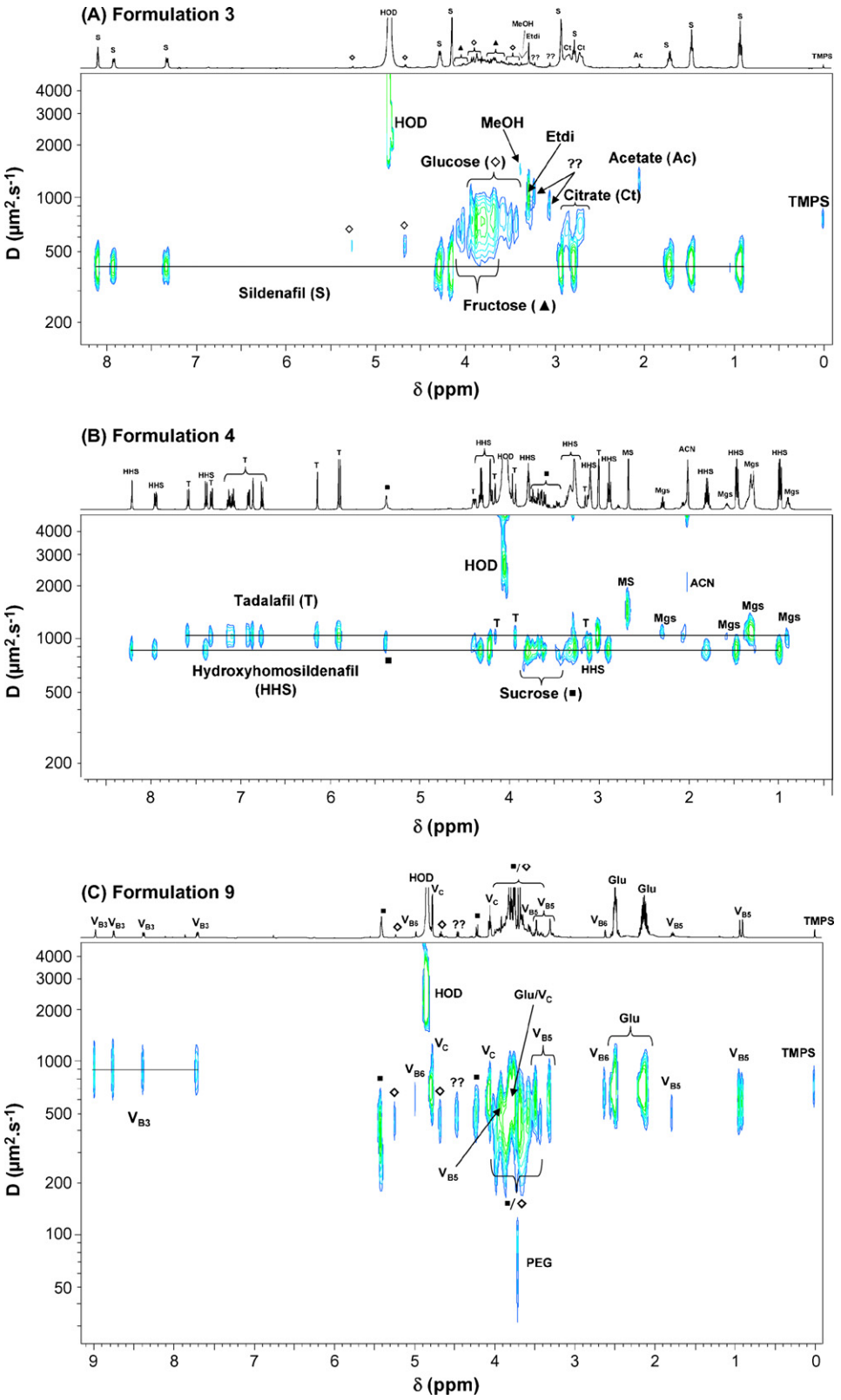


Fig. 1. 2D DOSY ^{1}H NMR spectra of formulations **3**, **4** and **9** (solvent CD_3CN:D_2O 80:20 for **4** and D_2O for **3** and **9**). S, sildenafil; T, tadalafil; HHS, hydroxyhomosildenafil; V_{B3}, vitamin B_3; V_{B5}, panthenol; V_{B6}, pyridoxine; V_C, ascorbic acid; (▲) fructose; (◊) glucose; (■) sucrose; Ct, citrate; Etdi, 1,2-ethanedisulfonate; Ac, acetate; Mgs, magnesium stearate; MS, methanesulfonate; Glu, glutamic acid; PEG, polyethylene glycol; MeOH, methanol; ACN, acetonitrile; TMPS, trimethylsilylpropane sulfonic acid (internal reference); (?) unknown.

Table 2
PDE-5 inhibitors or analogues and other ingredients detected by ^{1}H and 2D DOSY ^{1}H NMR in the 17 herbal products analyzed ((✚) detected in D_2O and (●) compound only detected in CD_3CN:D_2O (80:20)).

Formulations	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
PDE-5 inhibitors or analogues																	
sildenafil	✚	✚	✚		✚	✚											
tadalafil				●													●
vardenafil					✚												
hydroxyhomosildenafil				✚													
thiomethisosildenafil											●[a]						
Vitamins																	
niacin (Vit B_3)						✚				✚					✚		
nicotinamide (Vit B_3)									✚	✚							
ascorbic acid (Vit C)									✚								
panthenol (Vit B_5)									✚								
pyridoxine (Vit B_6)									✚								
Other actives																	
honokiol										✚							
yohimbine															✚	✚	
Sugars																	
lactose	✚																
glucose			✚	✚	✚			✚	✚	✚	✚	✚	✚	✚	✚	✚	✚
fructose			✚		✚		✚	✚			✚	✚	✚		✚		✚
sucrose				✚					✚	✚	✚			✚	✚	✚	
Amino acids																	
alanine															✚		
L-arginine															✚		
citrulline																✚	
GABA														✚			
glutamic acid									✚								
L-lysine																✚	
L-theanine										✚							
tyrosine																✚	
Other compounds																	
magnesium stearate	●			●	●	●			●		●			●	●	●	●
1,2-ethanedisulfonate			✚		✚												
methanesulfonate				✚	✚												
polyethylene glycol	✚								✚								
citrate	✚	✚	✚			✚											
glycerol		✚															
lactate			✚		✚										✚	✚	
taurine															✚	✚	
triacetin														✚			
acetate	✚	✚	✚		✚	✚											
ethanol	✚				✚												
methanol		✚	✚														

[a]Minor signals corresponding to thiosildenafil were also detected.

(10 mmol L^{-1}, pH 3). The flow rate was 0.6 mL min^{-1} and the volume injected 10 μL. A calibration curve was constructed from the analysis of four solutions containing pure tadalafil in a concentration range of 0.01–0.1 mg mL^{-1}. Each standard solution was injected in triplicate in the chromatographic system. The linearity ($R^2 > 0.999$) was evaluated by least-squares linear regression analysis.

For quantitative analysis of sildenafil, the mobile phase consisted of a volumetric mixture (50:50, v/v) of acetonitrile and a buffer solution (ammonium acetate 10 mmol L^{-1}, pH 7). A calibration curve was constructed from the analysis of four solutions containing pure sildenafil in a concentration range of 0.0012–0.05 mg mL^{-1}. Each standard solution was injected in triplicate in the chromatographic system. The linearity ($R^2 > 0.998$) was evaluated by least-squares linear regression analysis.

2.5. LC–MS analysis

The HPLC system used consisted of an Agilent 1100 series apparatus. An Applied System QTRAP triple quadrupole mass spectrometer, equipped with a Turbo Ion Spray (TIS) interface was used for detection. Both were controlled by an Agilent Analyst software (Version 1.4). The column temperature was 30 °C. The mobile phase consisted of a mixture (50:50, v/v) of acetonitrile and a buffer solution (ammonium acetate 10 mmol L^{-1}, pH 7). The flow rate was 0.6 mL min^{-1} and the injected volume 5 μL.

The mass spectrometer was operated in positive ionization mode with TIS heater set at 450 °C. Nitrogen served both as auxiliary, collision gas and nebulizer gas. The operating conditions for TIS interface were: (i) in MS mode: mass range 200–550 u (1 s), step size 0.1 u; Q1 TIS MS spectra were recorded in profile mode, IS 5000 V, DP 85 V; (ii) in MS–MS mode: precursor mass 489 u; mass range 10–500 u (0.35 s); step size 0.15 u; LC–MS–MS spectra were recorded in profile mode, IS 5000 V, DP 85 V, CE 40 V.

3. Results

3.1. Conventional ^{1}H NMR and 2D DOSY ^{1}H NMR analyses

All formulations were analyzed with 2D DOSY ^{1}H NMR. 2D DOSY spectra of formulations **3**, **4** and **9** along with their corresponding 1D spectrum are presented in Fig. 1. Each formulation was analyzed in two solvents, D_2O and CD_3CN:D_2O (80:20) in order to solubilize different components. All the peaks of a same compound are lined up, which allows a more accurate identification of various components in the complex mixtures.

All the ingredients detected by NMR investigations in the herbal drugs under study are reported in Table 2. Six active pharmaceutical ingredients (API) corresponding to PDE-5 inhibitors or analogues were observed: sildenafil in formulations **1**, **2**, **3** and **6**; sildenafil and vardenafil in formulation **5**; thiomethisosildenafil and a low amount of thiosildenafil in formulation **11**; tadalafil in formulation **17**; tadalafil and hydroxyhomosildenafil in formulation **4**. The spectral separation of these two actives according to their self-diffusion coefficients D is shown in Fig. 1. D generally decreases with increasing molecular weight (MW). The difference between the two MWs must be sufficient to get different Ds and so a separation of the signals. It is generally admitted that the ratio Δm/mean(MW) ($\Delta m = MW_A - MW_B$) must not be lower than 0.10–0.15 to observe separated signals along the diffusion axis. The MWs of tadalafil and hydroxyhomosildenafil are 389.4 and 504.6, respectively; the ratio Δm/mean(MW) is 0.26, which ensures a correct "virtual separation" of these two species in the DOSY spectrum. Indeed Ds measured for tadalafil and hydroxyhomosildenafil are 1030 and 860 $\mu m^2\ s^{-1}$, respectively.

Table 3 reports the NMR spectral data of sildenafil, vardenafil, hydroxyhomosildenafil, thiomethisosildenafil and tadalafil, measured in D_2O or CD_3CN:D_2O (80:20) for tadalafil and thiomethisosildenafil. The ^{1}H NMR resonances were assigned by 2D NMR experiments (gCOSY, gHSQC and gHMBC) and comparisons with published NMR data [8,9,13,15,18–20].

To confirm the identification of hydroxyhomosildenafil, we used LC–MS–MS. The pseudo molecular ion $[M+H]^+$ was measured by LC–MS at m/z 505 and the LC–MS–MS spectrum then allowed an unambiguous structural elucidation. Indeed, hydroxyhomosildenafil produced characteristic fragment ions at m/z 487, 377, 283 and 99 [3,7]. Likewise, the presence of the new adulterant thiomethisosildenafil detected by NMR in formulation **11** was confirmed by MS–MS analysis. The pseudo molecular ion $[M+H]^+$ was measured at m/z 505 and produced characteristic fragment ions at m/z 448, 393, 327, 315, 299, 113 and 99 (Fig. 2A). Fragment ion at m/z 393 corresponds to a characteristic $[R–S(O)–OH_2]^+$ ion and the whole observed fragmentation pattern was in accordance with published data on sildenafil derivatives [27]. In formulation **11**, minor signals of thiosildenafil could also be detected by NMR, e.g. an aromatic doublet at 8.32 ppm (H_9) and a singlet at 2.69 ppm ($N–CH_3$). An additional experiment by MS–MS allowed to observe the pseudo molecular ion $[M+H]^+$ at m/z 491, which produced characteristic fragmentation ions at m/z 407, 341, 327 and 299 [19].

Other APIs were also identified. Honokiol used in traditional Japanese medicine as an anxiolytic, anti-thrombotic, anti-depressant, anti-emetic, and anti-bacterial drug was found in formulation **10**; yohimbine, a drug for male impotence, in formulations **15** and **16**, and several vitamins (B_3, B_5, B_6 and C) in formulations **6**, **9**, **10** and **15**.

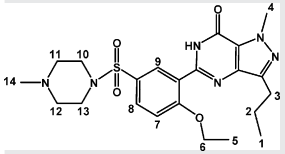
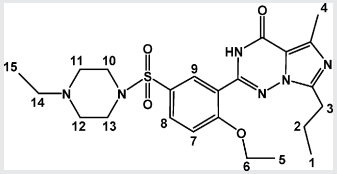
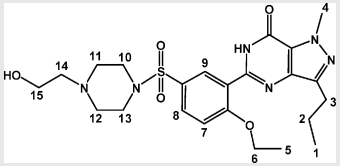
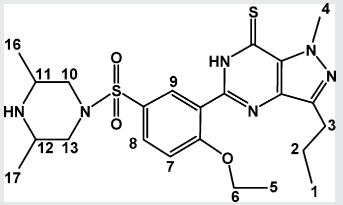
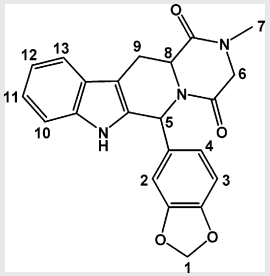
Various other components of tablets or capsules were detected, mainly sugars (lactose, glucose, fructose and sucrose) and amino acids (alanine, arginine, citrulline, GABA, glutamate, lysine, theanine and tyrosine) (Table 2). The lubricant magnesium stearate was present in ten out of the seventeen formulations. Two compounds with high diffusion coefficients (985 and 1200 $\mu m^2\ s^{-1}$), 1,2-ethanedisulfonate (Etdi) and methanesulfonate (MS), were observed in formulations **3** (Etdi), **4** (MS) and **5** (Etdi and MS) (Fig. 1A and B). Polyethylene glycol (PEG) used as lubricant in tablets is present in formulations **1** (Fig. 3A) and **9** (Fig. 1C); it gives a characteristic broad singlet at 3.74 ppm with a low diffusion coefficient (<100 $\mu m^2\ s^{-1}$). Additional signals of citrate, glycerol, lactate, taurine, triacetin, acetate, ethanol and methanol were detected in some formulations (Table 2).

The unambiguous identification of the components in herbal formulations was achieved by comparison of 1D ^{1}H NMR data of standards and addition of authentic standards. The chemical shifts and coupling constants of all ingredients, except PDE-5 inhibitors and analogues (Table 3), are reported in Table 4. Lactose, glucose and sucrose were easily detected by the signals of their anomeric protons giving characteristic unshielded doublets between 4 and 5.3 ppm. Magnesium stearate, honokiol and yohimbine were only detected in CD_3CN:D_2O while all other components were also observed in D_2O.

3.2. 3D DOSY–COSY ^{1}H NMR analysis

Fig. 3 illustrates the different NMR spectra obtained in the 3D DOSY–COSY ^{1}H NMR experiment applied to formulation **1**. First, the classical 2D DOSY (Fig. 3A) and COSY–DQF (Fig. 3B) spectra are shown. On the whole COSY spectrum diagonal signals and off-diagonal cross peaks of sildenafil, citrate, lactose and ethanol were observed. Other spectra (C–E) are those of the projections from the 3D DOSY-COSY experiment. The main

Table 3
^{1}H NMR characteristics of PDE-5 inhibitors and analogues detected in herbal formulations analyzed.

Sildenafil[a]			Vardenafil[b]			Hydroxyhomosildenafil[c]			Thiomethisosildenafil[d]			Tadalafil[e]		
														
δ (ppm)	Multiplicity[f] (J)	No.	δ (ppm)	Multiplicity[f,g]	No.	δ (ppm)	Multiplicity[f] (J)	No.	δ (ppm)	Multiplicity[f] (J)	No.	δ (ppm)	Multiplicity[f] (J)	No.
8.09	1H, d (2.4)	9	8.05	1H, d	9	8.10	1H, d (1.9)	9	8.43	1H, d (2.4)	9	7.57	1H, d (7.9)	13
7.94	1H, dd (8.7, 2.4)	8	7.95	1H, dd	8	7.91	1H, dd (8.8, 1.9)	8	7.95	1H, dd (8.8, 2.4)	8	7.30	1H, d (7.9)	10
7.35	1H, d (8.7)	7	7.33	1H, d	7	7.32	1H, d (8.8)	7	7.40	1H, d (8.8)	7	7.09	1H, m	11
4.29	2H, q (6.8)	6	4.29	2H, q	6	4.28	2H, q (7.0)	6	4.37	2H, q (7.0)	6	7.07	1H, m	12
4.18	3H, s	4				4.15	3H, s	4	4.47	3H, s	4	6.89	1H, dd (8.0, 1.4)	4
3.94, 3.28	4H, 2 broad s	10/13							3.89	2H, dd (11.4, 2.0)	10/13 eq	6.85	1H, d (1.4)	2
									2.43	2H, m	10/13 ax	6.74	1H, d (8.0)	3
2.82	2H, t (7.4)	3	2.75	2H, t	3				2.92	2H, t (7.5)	3	6.12	1H, s	5
			2.50	3H, s	4							5.89	1H, d (1.0)	1
3.62	2H, 1 broad s	11							3.38	2H, m	11/12	5.88	1H, d (1.0)	1′
						3.90	3H, t (4.9)	15				4.37	1H, dd (11.6, 4.0)	8
2.92	3H, s	14	2.90	2H, q	14	3.49	2H, q (9.8)	14				4.14	1H, dd (17.6, 1.4)	6
						3.45	8H, broad s	10/13/11/12				3.90	1H, d (17.6)	6’
						2.77	2H, t (7.4)	3				3.60	1H, dd (15.7, 4.2)	9
1.73	2H, sext (7.4)	2	1.76	2H, sext	2	1.72	2H, sext (7.4)	2	1.82	2H, sext (7.5)	2	3.11	1H, ddd (16.1, 11.8, 1.0)	9′
1.46	3H, t (7.0)	5	1.50	3H, t	5	1.48	3H, t (7.0)	5	1.55	3H, t (7.0)	5	2.97	3H, s	7
			1.32	3H, t	15									
									1.25	6H, d (6.5)	16/17			
0.93	3H, t (7.4)	1	0.94	3H, t	1	0.93	3H, t (7.2)	1	0.99	3H, t (7.4)	1			

[a] Data were measured in formulation **2** (D_2O, pH 3.3).
[b] Data were measured in formulation **5** (D_2O, pH 4.3).
[c] Data were measured in formulation **4** (D_2O, pH 4.8).
[d] Data were measured in formulation **11**.
[e] Data were measured in formulation **17** (CD_3CN:D_2O, 80:20).
[f] d: doublet; dd: doublet of doublet; ddd: doublet of doublet of doublet; s: singlet; m: multiplet; t: triplet; q: quadruplet; sext: sextuplet.
[g] Vardenafil coupling constants were not measured due to overlaps with sildenafil signals.

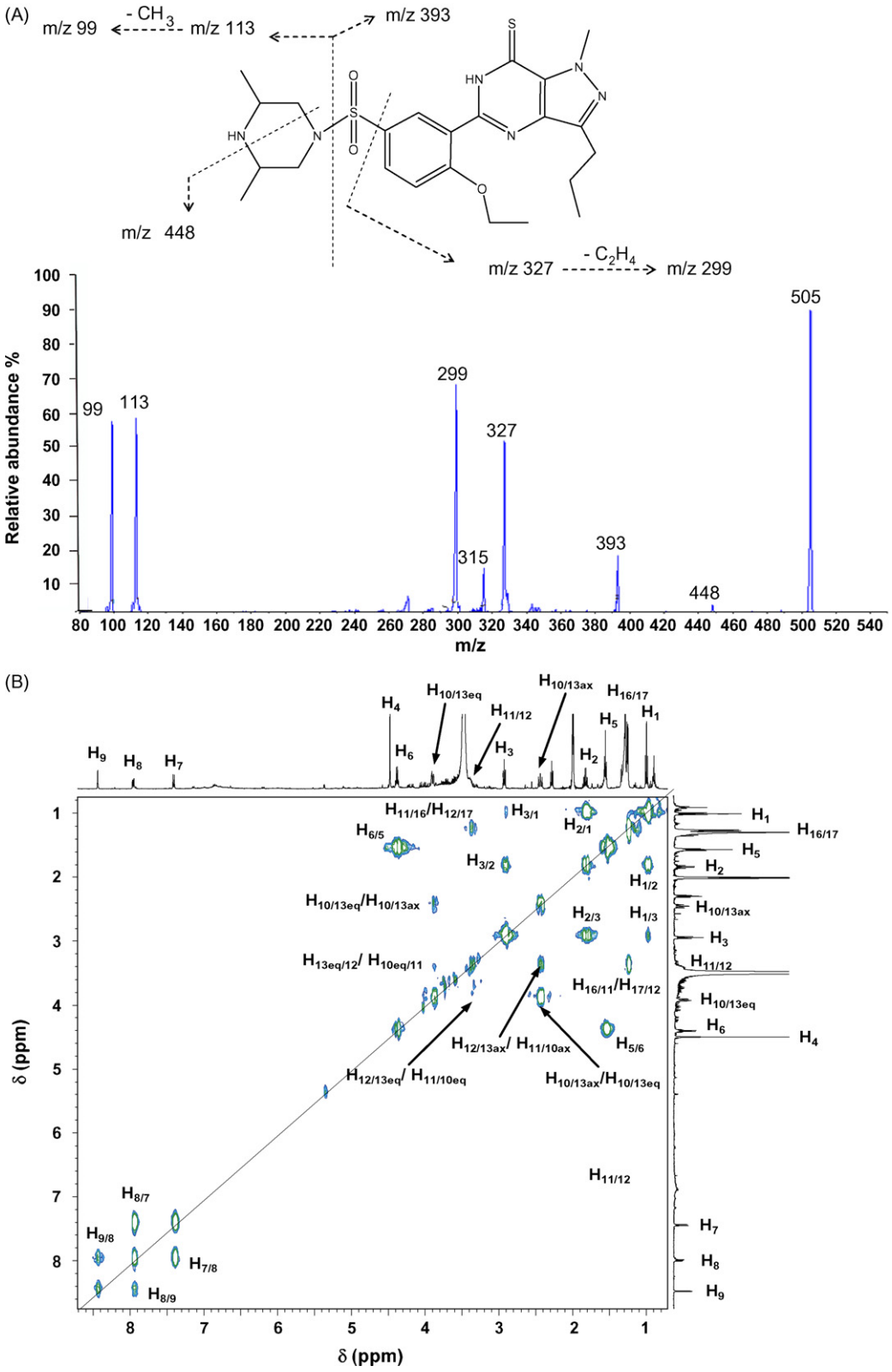


Fig. 2. MS and NMR spectra for the new adulterant thiomethisosildenafil detected by NMR in formulation **11**. (A) Positive mode MS–MS product ion scan of $[MH]^+$ ion at m/z 505 and fragmentation pathway proposed and (B) COSY extraction from 3D DOSY–COSY ^{1}H NMR experiment at $D = 870\ \mu m^2\ s^{-1}$.

interest of this 3D experiment is to extract the COSY spectrum of each component of the mixture from a selected line in the DOSY spectrum. For example, Fig. 3C shows the COSY projection of sildenafil at a diffusion coefficient (D) of 340 $\mu m^2\ s^{-1}$; typical cross peaks $H_{1/2}$, $H_{2/3}$, $H_{5/6}$ and $H_{7/8}$ were detected. Only some minor residual signals of lactose could be observed on-diagonal between 3.5 and 4.5 ppm. The COSY projection corresponding to $D = 510\ \mu m^2\ s^{-1}$ (Fig. 3D) shows exclusively the signals of lactose

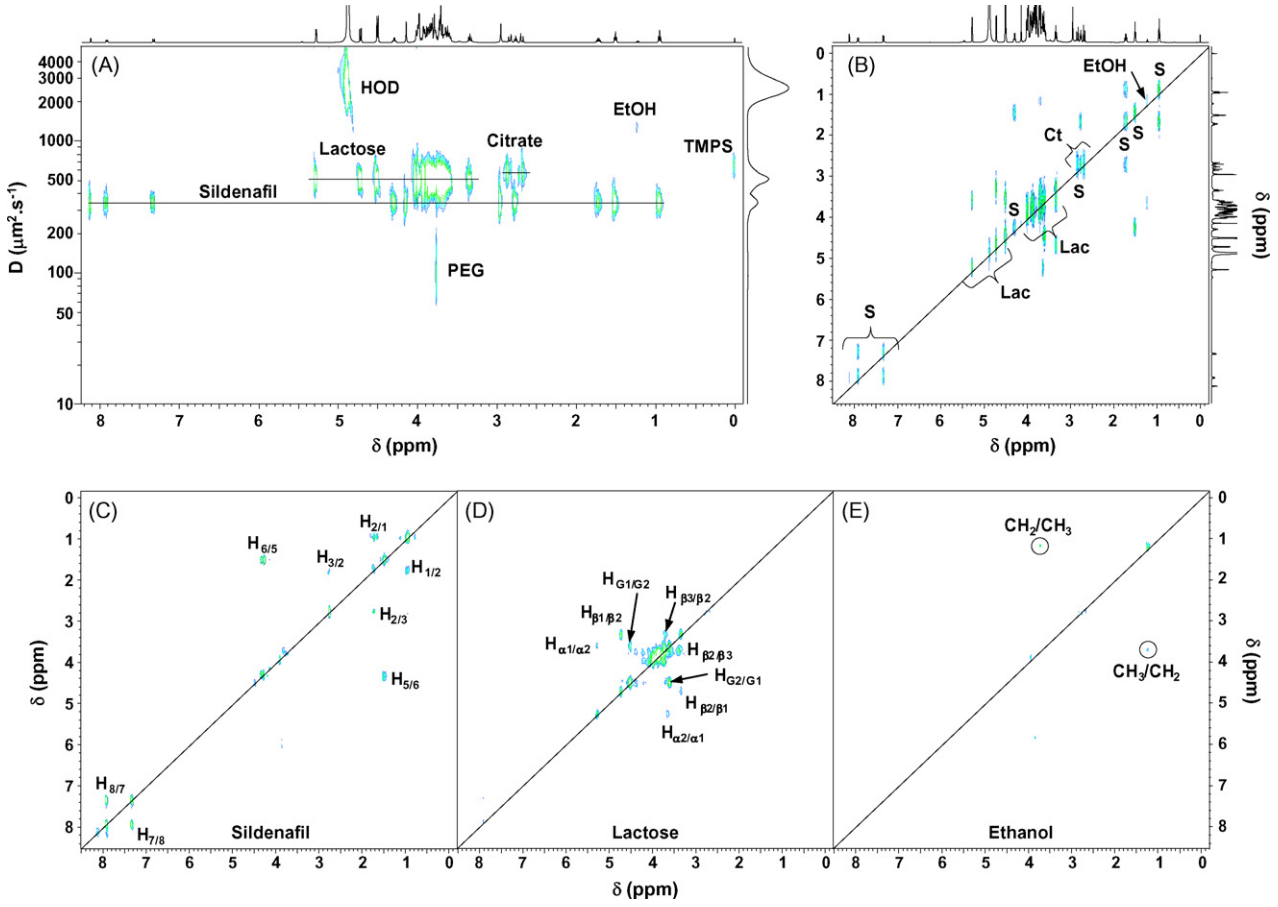


Fig. 3. NMR spectra of formulation **1** in D_2O. (A) 2D DOSY ^1H spectrum; (B) COSY–DQF spectrum; COSY extractions from 3D DOSY–COSY experiment at (C) $D = 340\ \mu m^2\ s^{-1}$, (D) $D = 510\ \mu m^2\ s^{-1}$, (E) $D = 1250\ \mu m^2\ s^{-1}$. S, sildenafil; Lac, Lactose; Ct, citrate; EtOH, ethanol; PEG, polyethylene glycol; TMPS, trimethylsilylpropane sulfonic acid (internal reference for chemical shift). In solution, lactose is an equilibrium mixture of α- and β-anomers, which explains the three anomeric proton resonances arising from the α- and β-anomers of glucose (marked H_α and H_β) while the galactose residue exists only in the β-form (marked G).

while that obtained at $D = 1250\ \mu m^2\ s^{-1}$ (Fig. 3E) corresponds to the signals of ethanol with some residual on-diagonal signals of citrate around 2.8 ppm. Fig. 2B shows the COSY projection corresponding to $D = 870\ \mu m^2\ s^{-1}$) of the 3D DOSY–COSY experiment of formulation **11** containing the newly identified compound thiomethisosildenafil. These spectra clearly highlight the interest of a 3D experiment as virtual separation provided by DOSY acquisition can lead to real structural determination by extraction of COSY spectra.

3.3. Quantification of PDE-5 and analogues

The contents of PDE-5 inhibitors or analogues were measured by HPLC for sildenafil and tadalafil or by NMR for vardenafil, hydroxyhomosildenafil, thiomethisosildenafil and thiosildenafil. Eight (**1**–**6**, **11**, **17**) out of the 17 herbal formulations contained at least one active ingredient. Formulations **1**, **2**, **3** and **6** contained sildenafil alone at 107 ± 7, 107 ± 10, 120 ± 2 and 0.3 ± 0.2 mg/tablet (or capsule), respectively. Formulation **4** contained tadalafil (31 ± 1 mg) and hydroxyhomosildenafil (48 ± 1 mg). Formulation **5** also contained two actives, sildenafil (58 ± 3 mg) and vardenafil (16 ± 1 mg). 10 ± 1 mg of tadalafil were measured in formulation **17**. Formulation **11** contained 15 ± 1 mg of thiomethisosildenafil; the amount of thiosildenafil was estimated on the aromatic H_9 signal to be about 5% of thiomethisosildenafil, i.e. ~0.75 mg.

4. Discussion

The contents of 17 different herbal drugs or dietary supplements for erectile dysfunction have been determined by the use of 2D DOSY ^1H NMR. Eight formulations (**1**–**6**, **11** and **17**) were found to be adulterated with synthetic PDE-5 inhibitors or analogues. The characterization of these compounds is generally carried out by HPLC or LC–MS–MS [4,7,10,12,14]. However, 2D DOSY ^1H NMR has the advantage of allowing the detection of various components of complex mixtures in a single run. With this method, we were able to identify the three PDE-5 inhibitors that have been approved by the US and European health authorities, i.e. sildenafil (Viagra®), vardenafil (Levitra®) and tadalafil (Cialis®), but also hydroxyhomosildenafil first described in 2004 by Blok-Tip et al. [3], and another analogue never reported in the literature, thiomethisosildenafil. In addition to these active ingredients, about 30 compounds or excipients were characterized (Table 2).

Up to 2004 only three analogues had been identified in herbal drugs or dietary supplements, homosildenafil [1,3,4], acetildenafil [2,3], and hydroxyhomosildenafil [3]. Since 2005 herbal drugs marketed for sexual dysfunction have become the target of increasing adulterations and today 14 adulterant PDE-5 analogues (including thiomethisosildenafil described for the first time in this study) have been characterized (Table 5). All these analogues are structurally close to parent compounds as modifications involve minor changes by substitution of functional groups, i.e. ethyl for methyl,

Table 4
^{1}H NMR characteristics of the main signals of ingredients found in herbal formulations (except PDE-5 inhibitors and analogues listed in Table 3).

Compound	^{1}H NMR δ (ppm)[a]; multiplicity[b] (J, Hz)
Vitamins	
Niacin (Vit B_3)	8.95 dd (2.2, 0.8), 8.63 dd (5.0, 1.5), 8.35 dt (8.0, 2.0), 7.60 ddd (8.0, 4.9, 0.8)
Nicotinamide (Vit B_3)	8.89 dd (2.2, 0.8), 8.69 dd (5.0, 1.5), 8.21 dt (8.0, 2.0), 7.57 ddd (8.0, 4.9, 0.8)
Ascorbic acid (Vit C)	4.77 d (1.8), 4.05 ddd (7.9, 6.1, 1.8), 3.75 m
Panthenol (Vit B_5)	3.91 s, 3.65 t (6.5), 3.45 AB (11.2), 3.29 m, 1.77 quin (6.6)
Pyridoxine (Vit B_6)	8.10 s, 4.97 s, 4.79 s, 2.61 s
Other actives	
Honokiol*	7.23 s, 7.21 d (2.2), 7.03 d (2.2), 6.97 d (2.2), 6.95 d (2.2), 6.92 m, 6.00 m, 5.04 m, 3.37 d (6.5), 3.31 d (6.5)
Yohimbine*	7.51 d (8.0), 7.40 d (8.0), 7.19 t (7.4), 7.10 t (7.4), 4.28 q (2.7), 3.49 m, 3.13 m, 2.73 m, 2.44 dd (11.5, 2.5), 2.21 m, 1.92 dt (14.2, 3.1), 1.72 m, 1.49 m
Sugars	
Lactose	5.27 d (3.8), 4.71 d (7.8), 4.49 d (7.8), 4.01-3.57 m, 3.33 t (8.4)
Glucose	5.25 d (3.8), 4.66 d (8.1), 3.89-3.36 m, 2.99 t (8.4)
Fructose	4.06-4.01 m, 3.72-3.65 m, 3.60-3.55 m
Sucrose	5.44 d (3.8), 4.24 d (9.0), 4.08 t (8.4), 3.89-3.57 m, 3.29 t (8.4)
Amino acids	
Alanine	3.50 q (7.2), 1.49 d (7.2)
L-Arginine	3.78 t (6.1), 3.26 t (6.9), 1.93 m, 1.69 m
Citrulline	3.77 t (6.2), 3.15 dt (6.8, 1.8), 1.90 m, 1.52 m
GABA	2.99 t (7.7), 2.28 t (7.3), 1.88 quin (7.6)
Glutamic acid	3.79 dd (7.2, 5.2), 2.49 t (7.9), 2.13 m
L-Lysine	3.85 t (6.5), 3.03 t (7.5), 1.80 m, 1.60 m
L-Theanine	3.78 t (6.1), 3.20 q (7.2), 2.40 m, 2.14 q (7.2), 1.11 t (7.2)
Tyrosine	7.19 d (8.3), 6.90 d (8.3), 3.83 dd (7.8, 5.2), 3.09 ABX (14.5, 7.9, 5.0)
Other compounds	
Magnesium stearate*	2.28 t (7.5), 1.57 quin (6.8), 1.28 broad s, 0.89 t (3.1)
1,2-Ethanedisulfonate	3.30 s
Methanesulfonate	2.84 s
Polyethylene glycol	3.74 broad s
Citrate	2.76 AB (16.0)
Glycerol	3.79 m, 3.61 ABX (11.9, 6.2, 4.3)
Lactate	4.10 q (7.0), 1.34 d (7.0)
Taurine	3.43 t (6.6), 3.27 t (6.6)
Triacetin	5.31 m, 4.33 ABX (12.2, 5.8, 4.0), 2.12 s, 2.10 s

[a] Spectra were recorded in D_2O except for compounds marked with an asterisk (*) recorded in CD_3CN:D_2O, 80:20.
[b] s: singlet; d: doublet; dd: doublet of doublet; ddd: doublet of doublet of doublet; t: triplet; dt: doublet of triplet; m: multiplet; q: quadruplet; quin: quintuplet.

Table 5
Adulterants found in herbal products or dietary supplements marketed for sexual dysfunction reported in bibliography.

Preparation	Analytical methods	Active pharmaceutical ingredients detected	References
Functional food	HPLC-DAD, 1D, 2D NMR	Homosildenafil	[1]
Herb drinks	HPLC, 1D, 2D NMR	Acetildenafil	[2]
Herbal product	LC–MS, IR, NMR	Homosildenafil, acetildenafil, hydroxyhomosildenafil	[3]
Dietary supplement and herbal matrices	LC–ESI-MS, HPLC-UV	Sildenafil, tadalafil, Homosildenafil	[4]
Dietary supplement	HPLC-UV–ESI-MS	Sildenafil, tadalafil, vardenafil	[5]
Herbal product	LC–MS	Sildenafil, tadalafil	[6]
Dietary supplements	LC–DAD, LC–ESI-MS/MS	Sildenafil, tadalafil, vardenafil, homosildenafil, acetildenafil, hydroxyhomosildenafil	[7]
Herbal product	LC-UV, ESI-MS/MS, IR, NMR	Aminotadalafil, hydroxyhomosildenafil	[8]
Dietary supplement	LC–MS–MS, IR, 1D, 2D NMR	Sildenafil, homosildenafil, acetildenafil	[9]
Herbal dietary supplement	LC–MS	Piperidinovardenafil	[10]
Herbal matrices	LS–ESI-MS, FT-ICR-MS	Aminotadalafil, piperidinovardenafil, hydroxyacetildenafil, piperidinoacetildenafil	[11]
Herbal product	ESI-MS–MS	Sildenafil, tadalafil	[12]
Dietary supplement	HPLC–DAD, IR, LC–MS, NMR	Piperidinovardenafil, hydroxyacetildenafil	[13]
Herbal dietary supplement	LC–MS, UV, MS–MS, NMR	Methisosildenafil	[14]
Herbal dietary supplement	HPLC–DAD, MS^n	Nor-acetildenafil	[15]
Herbal product	HPLC–DAD, LC–MS–MS, ^{1}H NMR	Vardenafil hydrolysis product	[16]
Herbal aphrodisiacs	HPLC–DAD, NMR	Sildenafil, vardenafil, piperidinovardenafil, homosildenafil, acetildenafil, hydroxyhomosildenafil	[17]
Herbal aphrodisiacs	LC–MS, UV and IR, MS–MS, NMR	Thiohomosildenafil	[18]
Dietary supplement	LC-UV, high resolution MS, ESI-MS/MS, NMR, IR	Benzamidenafil	[19]
Health supplements	High resolution MS, ESI-MS/MS, NMR, UV	Thiohomosildenafil, thiosildenafil	[20]
Herbal drugs and dietary supplements	2D DOSY ^{1}H NMR	Sildenafil, tadalafil, vardenafil, hydroxyhomosildenafil, thiosildenafil, **thiomethisosildenafil**	This study

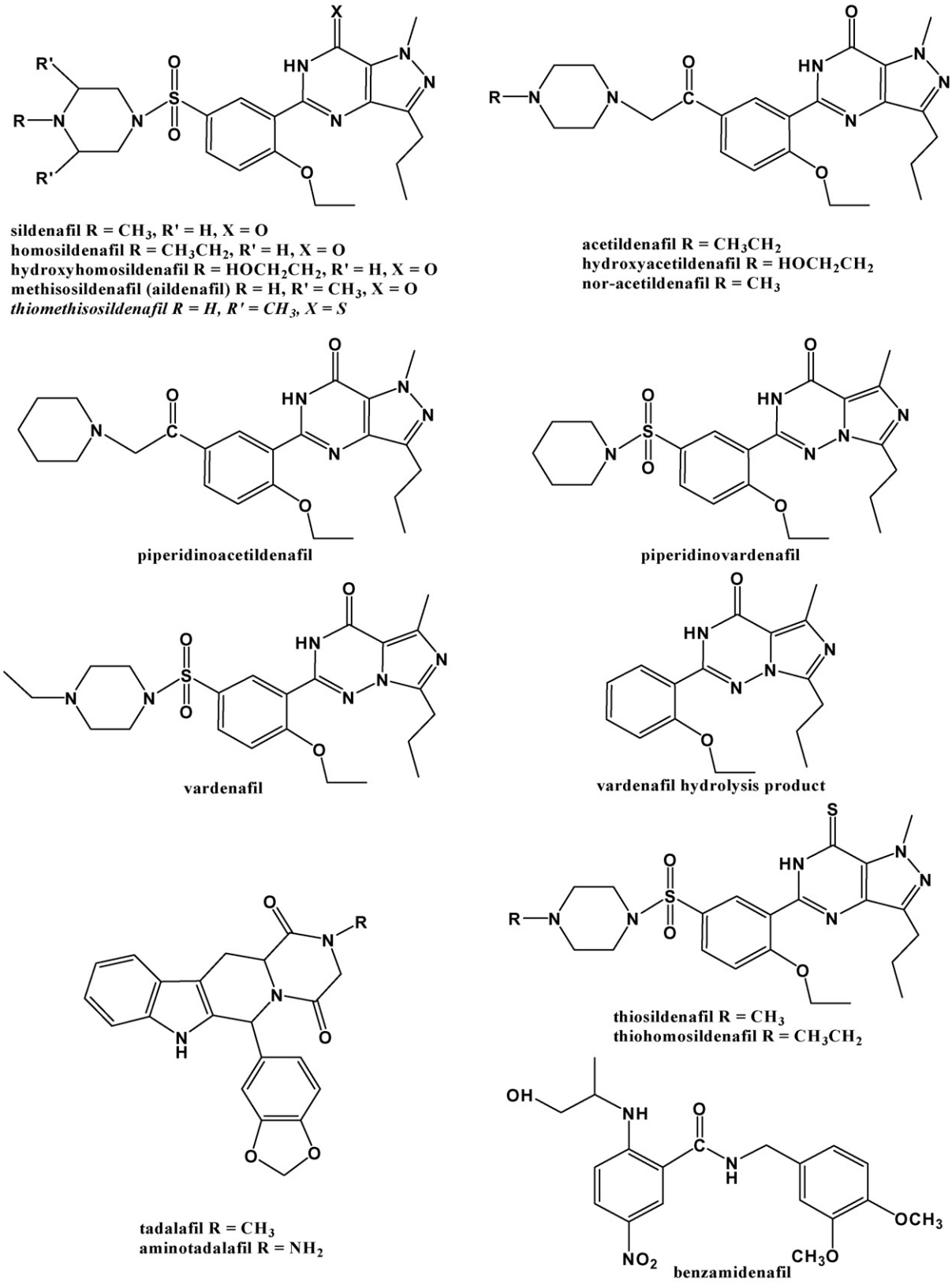


Fig. 4. Structures of PDE-5 inhibitors and adulterant analogues found in herbal products or dietary supplements.

acetyl for sulfonyl, thiocarbonyl for carbonyl, piperidine for N-ethylpiperazine, 2,6 dimethylpiperazine for N-methylpiperazine (Fig. 4). Such analogues are difficult to detect with routine chromatographic screening methods because of various structural modifications, and they might be used in an attempt to evade regulatory inspection [28]. This emphasizes the need for non-selective analytical methods that do not require any prior knowledge of the structure of the molecules.

The adulterated formulations analyzed in this study, manufactured in China, Taiwan, Estonia and Spain, were advertised as "all natural" or containing only vitamins. If the amount of sildenafil found in formulation **6** is low (0.3 ± 0.2 mg/capsule), it is greater than 100 mg in formulations **1**, **2** and **3**, which is high as the usual dose of sildenafil is 50 mg and the maximal recommended dose 100 mg. Formulation **3** claims on the blister packaging that it contains "no sildenafil citrate" whereas 120 mg per capsule were found. Formulation **4** contains 31 mg of tadalafil that is three times the normal starting dose, as well as hydroxyhomosildenafil for which toxicological data are not known or available yet. The amount of vardenafil in formulation **5** (16 mg) is around 1.5 times the starting dose for this drug. To the best of our knowledge, pharmacological and toxicological data are not documented for thiomethisosildenafil and thiosildenafil found in formulation **11**.

This study is the first reporting the use of 2D DOSY and 3D DOSY–COSY ^{1}H NMR for the analysis of herbal drugs. Methodological 3D DOSY–COSY studies have already been described, with application to model mixtures of alcohols [29], amino acids [30] or oligonucleotides [24]. In the last study, a DOSY–COSY example on natural beeswax as a complex medium was also reported, but no structural attribution of the mixture was done. In the present study, the 3D DOSY–COSY experiment provided structural data as it allows to observe COSY subspectra for each component (Fig. 3), namely sildenafil, lactose, ethanol, and citrate (not shown).

5. Conclusion

This study presents a new application of 2D DOSY ^{1}H NMR for the analysis of herbal drugs or dietary supplements, which are undeniably complex mixtures. Eight formulations marketed for sexual dysfunction were adulterated with PDE-5 inhibitors or analogues. This work is, to the best of our knowledge, the first report about adulteration with thiomethisosildenafil. Adulteration of herbal medicines with undeclared synthetic drugs is a common and dangerous phenomenon and methods that ensure the quality and safety of these products have thus to be developed. In this context, 2D DOSY ^{1}H NMR spectroscopy is a powerful method for providing a multivariate fingerprint of a complex mixture especially in situations where the identity of the components is not known beforehand. The technique should now be considered as a useful and complementary tool among a standard 2D NMR analytical package. Moreover, its evolution towards 3D DOSY–COSY experiments noticeably increases its interest as structural data are easily obtained.

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