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Isolation and identification of ten new sildenafil derivatives in an alleged herbal supplement for sexual enhancement

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Abstract

A sexual enhancer dietary supplement in pre-commercialization phase was analyzed. It contained the two phosphodiesterase-5 inhibitors (PDE-5i) sildenafil and methisosildenafil as major adulterants. Fourteen more sildenafil derivatives were detected and after isolation, their structures were elucidated thanks to NMR, high resolution and tandem mass spectrometry, and UV spectroscopy. Ten of them were never described. All these compounds are probably by-products of different reaction steps during the synthesis of the two PDE-5i that were not properly eliminated during the purification procedure. The total amount of sildenafil-related compounds was estimated at 68 mg per capsule, sildenafil and methisosildenafil accounting for 20 mg and 38 mg respectively.

Keywords: SFC; HPLC; NMR; mass spectrometry; adulteration; dietary supplement;

1. Introduction

Nowadays, consumers' keen interest in plant food supplements stems from their sometimes inaccurate perception that these products are safe because of their natural origin. In the absence of strict and uniform regulation and control of these products, unscrupulous manufacturers may fraudulently add active substances to these herbal preparations to increase the desired pharmacological effect. The phenomenon of adulteration of sexual enhancer dietary supplements with synthetic phosphodiesterase-type 5 inhibitors (PDE-5i), whether approved (sildenafil, tadalafil, vardenafil) or unapproved (their analogues), has been widely documented [1-4]. Since the detection of homosildenafil, the first analogue reported in literature in 2003 as adulterant in a dietary supplement [5], their number has regularly increased to 80 in March 2017 [6] and continues to increase, as evidenced by recent studies [see for example 7-9].

In the present study, sildenafil and fifteen analogues were isolated from a dietary supplement intended for marketing, using Supercritical Fluid Chromatography (SFC) and Liquid Chromatography (LC) purification methods. All of the compounds were then fully identified by means of ultraviolet (UV) spectroscopy, mass spectrometry (MS), and ^1H and ^{13}C nuclear magnetic resonance (NMR). To our knowledge, ten of these compounds are new and have never been reported in literature.

2. Experimental

2.1. Materials

One dietary supplement in pre-commercialization phase was submitted for analysis. Its composition was claimed as follows: raspberry (*Rubus idaeus*) 25%, Solomon's seal (*Polygonatum sibiricum*) rhizome 25%, common yam (*Dioscorea opposita*) rhizome 20%, barberry wolfberry (*Lycium chinense*) fruit 15%, cassia (*Cinnamomum cassia*) bark 15%. Twenty-four capsules containing each 360 ± 20 mg of brown powder were used for this study.

All chromatographic solvents (RS-HPLC-Preparative grade for purification steps with preparative HPLC and RS-HPLC-GOLD-Ultra gradient grade for analytical purposes) were purchased from Carlo Erba (27100 Val de Reuil, France). Authentic standards of sildenafil and methisosildenafil were supplied by TRC (North York, ON, Canada). All other chemicals and reagents used as well as the NMR reference for internal chemical shift and quantification (sodium 2,2,3,3-tetradeutero-3-(trimethylsilyl) propanoate (TSP)) were supplied by Sigma Aldrich (St. Louis, MO, USA). Deuterated solvents were obtained from Euriso-Top (91194 Saint Aubin, France).

2.2. Purification of active compounds

2.2.1. Sample preparation prior to SFC experiments

A preliminary analysis showed that the sample contained a sort of tar which could prevent purification or complicate further purification steps. To get rid of it, a first pre-extraction procedure was implemented. The powder from 1 capsule was treated in two steps. It was first extracted with 3 mL of a CH₃CN:H₂O (80:20 v/v) mixture, vortexed for 15 s, sonicated for 10 min and centrifuged (3000 rpm) for 5 min at room temperature. The supernatant (2800 µL) was collected and transferred into a glass tube. In a second step, the pellet was re-extracted with the same protocol using 1 mL of solvent. The supernatants were then pooled and the solution was evaporated to dryness. This procedure was repeated for 24 capsules. For SFC purification, 2.9 g of the residual powder were dissolved by sonication in 58 mL of methanol. Before injection, samples were filtered on 0.45 µm GHP membranes.

2.2.2. Preparative SFC

All preparative SFC separations were carried out on a BetaSil Diol-100 (250 x 21.2 mm, 5 µm) column using a Berger Multigram II Preparative SFC system (Mettler Toledo, Viroflay,

France) and the SFC ProNTO software. Preparative SFC was operated with an elution gradient at a back pressure of 100 bar, a temperature of 40°C, a flow rate of 42.5 mL min⁻¹ and a UV detection at 235 nm. The gradient started by an isocratic elution of CO₂ and 10% methanol containing 0.5% (v/v) isopropylamine during 0.5 min, and the organic modifier percentage was then increased to 28% at 8 min and remained at this value until 15 min. At last the column was re-equilibrated in the initial elution conditions from 16 to 20 min (Table 1). 116 injections of 25 mg of raw extract dissolved in 500 µL of methanol were done. Seven fractions were collected as indicated in the chromatogram of Fig. 1A, i.e. 3-6 min (A), 6.05-6.45 min (B), 6.5-7.8 min (C), 7.85-8.75 min (D), 8.8-10.1 min (E), 10.15-11.45 min (F) and 11.5-13.2 min (G). All fractions were then evaporated to dryness.

2.2.3. Preparative LC

Each SFC fraction was dissolved either in the starting LC eluent (D, E, F, G) or in a mixture of the starting LC eluent and methanol (B, C) or acetonitrile (A) (Table 1). Purifications were performed on a Waters Prep 150 LC System instrument (Waters Corporation, Milford, MA, USA) equipped with a 2545 Binary Gradient pump, a 2707 autosampler, a 2998 photodiode array (PDA) detector and a WFC III fraction collector. Data were processed using the Chromscope software. Columns and elution conditions for preparative LC are reported in Table 1 and chromatograms are shown in Fig. 1B. UV detection was set at 235 nm for all experiments. Two compounds (**15** and **16**) were collected in the same fraction. All fractions recovered were lyophilized.

2.2.4. Analytical Ultra High Performance Liquid Chromatography (UHPLC)

Each LC fraction was dissolved in methanol at a concentration of ≈0.5 mg/mL and its purity was controlled by UHPLC using a Waters Acquity UHPLC system equipped with a

binary solvent delivery pump, an auto sampler, a PDA detector, a QDa mass detector and the Empower 3 software. The analytical conditions are reported in Table 1. UV spectra were obtained from these UHPLC analyses.

2.3. NMR and MS characterization of the dietary supplement and the purified compounds

2.3.1. NMR

Two to 7 mg of each lyophilized final fraction were solubilized in 1 mL of a CD₃CN:D₂O (80:20 v/v) mixture, and the solution was vortexed for 15 s and sonicated for 10 min. To achieve the solubilisation of compounds **9** and **14**, 5 µL of a 0.5 M NaOD solution were added. Thirty µL of a 5 mM solution of TSP in D₂O as an internal reference for chemical shift (δ) measurement were added before the NMR analysis.

For the quantitative determination of sildenafil derivatives in the dietary supplement (done in triplicate), around 10 mg of powder were accurately weighed and 1 mL of deuterated methanol (CD₃OD) was added. The suspension was submitted to vortex agitation for 15 s, sonication for 10 min, magnetic stirring for 20 min and it was then centrifuged (5 min, 3000 rpm). Thirty µL of a 5 mM solution of TSP were added to 800 µL of supernatant and the solution was transferred into a 5 mm NMR tube.

NMR spectra were recorded on a Bruker Avance 500 spectrometer (Bruker Biospin AG, Fallanden, Switzerland) equipped with a 5 mm cryoprobe at 298 K. The structural elucidation of purified sildenafil derivatives was achieved thanks to one-dimensional (1D; ¹H and ¹³C) and two-dimensional (2D; gCOSY, gHSQC, gHMBC and NOESY) experiments. The 1D ¹H NMR spectra of the dietary supplement were acquired with inverse gated decoupling for ¹³C using a GARP sequence. Acquisition parameters were as follows: number of scans 32, pulse width 10.7 µs (flip angle 90°), acquisition time 1.56 s, spectral width 10500 Hz, 32K data points, and relaxation delay 4 s; the recording time was thus ≈4 min. The NMR assignment of sildenafil

derivatives in the dietary supplement was done by adding successively the previously purified and identified compounds. For quantitative NMR experiments, the relaxation delay was lengthened to 15 s for a full relaxation of the ^1H resonances and the number of scans was raised to 128. NMR data were processed using the TOPSPIN 3.1 software.

2.3.2. High-Resolution Mass Spectrometry (HRMS)

Each lyophilized final fraction was dissolved in $\text{CH}_3\text{CN}:\text{H}_2\text{O}$ (80:20 v/v) and analyzed after direct infusion using a Waters XEVO G2 QTOF mass spectrometer with electrospray ionization in positive (ESI^+) and negative (ESI^-) modes. For both modes, the instrument parameters were as follows: for MS analysis, cone voltage 30 V, source temperature 110°C , desolvation temperature 300°C , cone gas flow rate 20 L h^{-1} , scan range m/z 50-1200; for MS/MS analysis, three different collision energies (15, 25 and 35 V) were applied with the cone voltage maintained at 30 V and the spectra were acquired with a mass precursor ion selection of 3 Da. All analyses were performed using the lockspray, which ensured accuracy and reproducibility. Leucine enkephalin ($1\text{ ng }\mu\text{L}^{-1}$) introduced by a lockspray at $3\text{ }\mu\text{L min}^{-1}$ was used as the lockmass generating reference ions at m/z 556.2771 or 554.2615 in positive or negative mode respectively. The MassLynx software was used to calculate the most probable chemical formula and the theoretical exact mass of the molecular and fragment ions by comparison with their measured accurate ionic masses. The elemental formula of each ion was confirmed by the agreement between the experimental and calculated values within a relative mass error (RME) $<3\text{ ppm}$ or between 3 and 5 ppm for respectively 93% and 7% of ions with

a molecular weight >100 Da and <5 ppm or between 5 and 10 ppm for 69% and 31% of ions with a molecular weight <100 Da.

2.4. Molecular modelling

The chemical structures were sketched (hybridization, hydrogenation, some geometry optimizations) using ChemAxon Marvin 17.25 (2017, ChemAxon, <http://www.chemaxon.com>). In order to generate a coarse-grained conformational sampling, compounds **1-5** were submitted to the calculation plugins (conformers, molecular dynamics) of Marvin using standard parameter sets. The structures were merged in SDF libraries using in-house software and imported in BIOVIA Discovery Studio Client (DSV) release 2016, Dassault Systèmes (<https://www.3dsbiovia.com/>) software. DSV was used to align molecules using a reference group and to measure interatomic distances for structures of interest (i.e. lowest energy conformers or frames). Molecular graphics were also produced using DSV.

3. Results

3.1. Preliminary ¹H NMR analysis of the dietary supplement

As soon as the dietary supplement was received for control in our lab, its ¹H NMR analysis was performed as usually [1] in order to detect a possible adulteration. The ¹H NMR spectrum clearly showed that the herbal mixture was not natural as expected from its claimed composition. The presence of two main contaminants could be deduced from the highest signals that were assigned to sildenafil (**12**) and methisosildenafil (**1**) thanks to our in-house NMR database. Nonetheless, our attention was caught by the numerous minor signals whose multiplicity and chemical shift could be compatible with those of PDE-5i analogues. To get rid of carbon satellites that increase spectrum complexity, a ¹³C GARP broadband decoupled ¹H NMR spectrum was acquired, which confirmed the presence of minor adulterants (Fig. 2).

In order to determine their structures, the dietary supplement was purified and the compounds detected were isolated. The main issue encountered in the purification of this complex mixture was the huge concentration differences of the various analytes as illustrated in the NMR spectrum where large and small signals co-exist. To overcome this difficulty and after some unsuccessful attempts using both reverse and normal LC (results not shown), we chose to work in two steps using a first SFC pre-fractionation followed by LC purifications.

3.2. Purification of the dietary supplement

The SFC purification was done using a BetaSil diol phase. This polar stationary phase and the chosen elution mode provided sharp enough peaks and satisfactory chromatographic resolution and retention times. The use of basic elution conditions allowed the reduction of peak tailing for molecules containing amine groups. Seven fractions were collected from 3 to 13.2 min as shown in [Fig. 1A](#).

Each SFC fraction was then purified by preparative LC. All experimental conditions are gathered in [Table 1](#) and chromatograms are illustrated in [Fig. 1B](#). The chromatographic conditions were chosen after a screening step using neutral or acidic medium and two stationary phases Kromasil C18 and CSH C18. The elution gradient was optimized for each separation.

The two steps of purification with polar (SFC) and non-polar (LC) chromatographic phases led to the purification of 16 compounds. The purity of each preparative LC fraction (except that containing compounds **15** and **16** which could not be separated) was evaluated from the UHPLC-UV chromatograms performed in routine analytical conditions and was in the range 88.8-99.3% (mean 96.3%). It can be noticed that 14 products eluted between 2.07 and 2.92 min ([Table 2](#)), which also justifies the use of two orthogonal chromatographic methods that provided complementary selectivity to achieve the purification.

3.3. Characterization of purified compounds

The chemical structures of the two major adulterants methisosildenafil (**1**) and sildenafil (**12**) deduced from the ^1H NMR analysis of the raw dietary supplement (*vide supra*) were confirmed by their UV ($\lambda_{\text{max}} \approx 225$ and 295 nm [10]), HRMS ($[\text{M}+\text{H}]^+$ 489.2277 and 475.3126 respectively) and 1D and 2D NMR spectra (Fig. 3, Tables 3-5). Some clues on the structures of the other isolated compounds could be deduced from their UV and HRMS characteristics.

The UV spectrum of compound **2** with the same profile and λ_{max} than those of compounds **1** and **12** indicated that its structure also displays the pyrazolo[4,3-d]pyrimidine-7-one ethoxyphenyl (or propoxyphenyl) sulfonamide moiety [10]. By comparison with the UV profiles of the 64 selected PDE-5i reported by the USP [10], it can be excluded that the chromophore of the other compounds is that of pyrazolopyrimidine-7-thione sulfonamide analogues, pyrazolopyrimidine-7-one or -7-thione with acetyl moieties, or of tadalafil or vardenafil derivatives. Nevertheless, similar chromophoric skeletons are observed for compounds **4/5/13**, **6/7** and to a lesser extent **3**, **8/9/14**, and **10/11** (Fig. 3).

3.3.1. MS and MS/MS analysis

The ESI^+ accurate mass data of parent and fragment ions of all the isolated compounds are gathered in Table 3. Some characteristic ESI^+ MS/MS spectra together with the structure of fragment ions are illustrated in Fig. 4A. The MS/MS fragmentations show the presence of a small (or very small) peak at m/z 166.0973-166.0985 corresponding to the molecular formula $\text{C}_8\text{H}_{12}\text{N}_3\text{O}^+$ whose proposed structure (Fig. 4B) is characteristic of the fragmentation of the pyrazolo[4,3-d]pyrimidine-7-one moiety of the sildenafil derivatives [11] and not of the isomeric vardenafil skeleton that leads to fragmentation ions at m/z 169 and 151 [12]. Moreover, a peak at m/z 299.1137-299.1151 corresponding to the molecular formula $\text{C}_{15}\text{H}_{15}\text{N}_4\text{O}_3^+$ is found in the fragmentation of all the compounds, except **15** and **16**. Its intensity

is markedly lower for compounds **2-7** and **13** but remarkably higher for compounds **8-11** and **14** than for methisosildenafil (**1**) and sildenafil (**12**). The structure of this ion (Fig. 4B and 4C) involves the occurrence of a pyrazolopyrimidine-7-one sulfonamide moiety (or that of the isomeric vardenafil structure which has already been excluded (see above)).

In comparison with the fragmentation profile of the dimethylpiperazine moiety of methisosildenafil **1** which generates major peaks at m/z 113, 99, 84 and 71 (the latter being characteristic of such a piperazine structure [13,14]), it can be stated that compounds **3, 4, 8** and **10** bear a dimethylpiperazine entity. Compounds **2, 5, 7, 9** and **11** display the same fragmentation peaks (except that the ion at m/z 99 is replaced by two ions at m/z 100 and 98) but also the presence of two intense additional ions at m/z 141 and 127 corresponding respectively to molecular formulae $C_8H_{17}N_2^+$ and $C_7H_{15}N_2^+$ (Fig. 4A and 4D for the proposed structures of the fragment ions). The increase of 28 and 14 m/z units with respect to the fragmentation ion of the dimethylpiperazine moiety at m/z 113 (molecular formula $C_6H_{13}N_2^+$) suggests the insertion of an ethyl group on the piperazine ring. Moreover, the presence of a product ion at m/z 72 (as intense as the ion at m/z 71), characteristic of the fragmentation of a N-ethylpiperazinyll group [13,14], is a good indication of the presence of a dimethyl-N-ethylpiperazine moiety. In comparison with the fragmentation of the N-methylpiperazine sulfonamide moiety of sildenafil **12** that gives peaks at m/z 163, 100, 99, 70 and 58 [12,13], it can be concluded that this entity is part of the structure of compounds **13** and **14** (Fig. 4A and 4D). The HRMS and MS/MS spectra of compounds **15** and **16** (not separated) do not show the presence of any fragmentation ion coming from piperazine or other amine substituent [13].

Compounds **2, 3, 4, 6** and **11** with a $[M+H]^+$ ion at m/z 517.2587-517.2600 suggesting the molecular formula $C_{25}H_{37}N_6O_4S^+$ are characterized by a gain of 28 mass units compared to methisosildenafil **1** at m/z 489.2277 ($C_{23}H_{33}N_6O_4S^+$), thus demonstrating the presence of a supplementary ethyl group. Compounds **5** and **7** with $[M+H]^+$ ions at m/z 545.2920 and

545.2922 suggesting the molecular formula $C_{27}H_{41}N_6O_4S^+$ are characterized by a gain of 56 mass units compared to methisosildenafil, thus indicating the presence of two additional ethyl groups. Because the HRMS/MS spectrum of compound **2** only shows product ions corresponding to methisosildenafil (m/z 489) and its characteristic fragment ions (m/z 432, 377, 311, 299, 283) as well as those of dimethyl-N-ethylpiperazine (Fig. 4B), it is therefore a methisosildenafil derivative with an ethyl group on the nitrogen atom of the piperazine ring (MSildEt^{N26}). MS/MS spectra of compounds **3-7** show (i) product ions corresponding to methisosildenafil and its fragment ions cited above, (ii) those of dimethyl or dimethyl-N-ethyl piperazine moiety, and (iii) additional peaks at m/z 460, 405, 339 (340 for compound **3**) and 327, i.e. 28 mass units greater than those at m/z 432, 377, 311 and 299, thus demonstrating that they all have an ethyl group located on the pyrazolopyrimidine part (Fig. 4B). As only tiny peaks at m/z 141, 127 and 72 are detected, it can be considered that compounds **3** and **4** do not have the ethyl group on the piperazine ring and therefore have it elsewhere, i.e. on the pyrazolopyrimidine skeleton. Compounds **5** and **7** have two ethyl groups, one on the piperazine moiety and the other on the pyrazolopyrimidine skeleton. Although its molecular mass is in agreement with the presence of only one additional ethyl group compared to methisosildenafil, the fragmentation of compound **6** is surprising because it shows the characteristic signals of ethyl groups on the piperazine ring (m/z 141, 127 and 72, although less intense than for **2**, **5** and **7** for which the presence of the ethyl group on the piperazine ring is well established), and on the pyrazolopyrimidine skeleton (m/z 460, 405, 339 and 327). A possible explanation of these observations is proposed below. The MS/MS spectrum of compound **11** shows major product ions at m/z 327, 311, 299 and 283, and less abundant peaks characteristic of the dimethyl-N-ethyl piperazine fragmentation at m/z 141, 127, 100, 98, 72 and 71 (Fig. 4C and 4D). The ions at m/z 299 and 283 result from the loss of a C_2H_4 moiety from ions at m/z 327 and 311, respectively. If ions at m/z 311, 299 and 283 are well-known fragments of

methisosildenafil, the ion at m/z 327 is only observed with compounds **3-7** for which it was unambiguously demonstrated (except for compound **6**) that the pyrazolopyrimidine skeleton bears an ethyl group. Compound **11** has thus two ethyl groups, one on the piperazine ring and the second on the pyrazolopyrimidine skeleton, which implies that its benzene ring is not substituted by an ethoxy group but by a hydroxyl group. Moreover, the absence of the ion at m/z 489 ($[M+H]^+$ of methisosildenafil) in the MS/MS spectrum, in contrast to what is observed with compounds **2-7**, confirms that the structure of compound **11** does not derive from that of methisosildenafil.

Compound **10** with the same $[M+H]^+$ molecular ion than methisosildenafil at m/z 489.2292 has the same fragments ions as compound **11** except that the piperazine product ions are those of a dimethylpiperazine entity. Therefore, it is most likely a methisosildenafil derivative with an ethyl group on the pyrazolopyrimidine ring and an OH substituent on the benzene ring. The $[M+H]^+$ molecular ion at m/z 461.1973 of compound **8**, corresponding to the molecular formula $C_{21}H_{29}N_6O_4S^+$, is 28 mass units lower than that of methisosildenafil ($C_{23}H_{33}N_6O_4S^+$). Its MS/MS spectrum shows prominent fragment ions at m/z 347, 299 and 283, and all the characteristic product ions of the dimethylpiperazine ring. The loss of this piperazine entity produces the fragment at m/z 347 and the further loss of SO_2 leads to the ion at m/z 283. As no fragment ions correspond to the loss of an ethyl group, it can be concluded that compound **8** is a methisosildenafil derivative with an OH instead of an ethoxy group on the benzene ring (MSildOH). Compound **9** displays the same molecular formula ($C_{23}H_{33}N_6O_4S^+$; $[M+H]^+$ 489.2292) than methisosildenafil, the same fragment ions at m/z 347, 299 and 283 than compound **8**, and the characteristic product ions of the dimethyl-N-ethylpiperazine moiety. It can therefore be stated that it is a methisosildenafil derivative with the ethoxy group on the benzene cycle replaced by an OH, and a dimethyl-N-ethylpiperazine entity (MSildOHEt^{N26}).

The molecular ions $[M+H]^+$ of compounds **13** (m/z 503) and **14** (m/z 447) are respectively 28 mass units higher and lower in comparison to that of sildenafil (**12**) at m/z 475. The MS/MS spectra of the two ions show the presence of N-methylpiperazine product ions (m/z 163, 100, 99, 70 and 58). This is a good indication that compound **13** bears an additional ethyl group on the sildenafil skeleton and shows that compound **14** is a sildenafil derivative whose ethoxy group on the benzene ring is replaced by a hydroxyl group (SildOH).

It should be noted that the ion at m/z 299 is much more intense in the MS/MS spectra of compounds with the benzene ring substituted by a hydroxyl (**8-11** and **14**) rather than by an ethoxy group (compare the spectrum from **11** to all other spectra presented in Fig. 4A), this observation being able to be considered as a good indication of the presence of a hydroxyl group on the benzene ring. Indeed, the ion at m/z 299 results from a well described fragmentation mechanism of sulfonamides involving a rearrangement of the SO_2 group resulting in the loss of SO [15]. But if the fragmentation of the ion at m/z 347 of MSildOH (**8**), MSildOHEt^{N26} (**9**) or SildOH (**14**) leads directly by this way to the ion at m/z 299, that of the ion at m/z 377 of methisosildenafil (**1**) or sildenafil (**12**) for example, requires a subsequent loss of C_2H_6 for the formation of the ion at m/z 299 [16] (Fig. 4B and 4C). The same is true for the ion at m/z 327 (299 + ethyl) which is more intense for compounds **10** and **11** than for compounds **3-7** (compare the spectrum of **11** with the spectrum of **4** in Fig. 4A).

Only a low amount of the mixture of compounds **15** and **16** was obtained after the various steps of purification and our attempts to separate them were unfruitful. The MS spectrum of the mixture shows two $[M+H]^+$ peaks, one very intense at m/z 313.1663 (compound **15**) and a second much less intense at m/z 341.1980 (compound **16**), corresponding respectively to molecular formulae $C_{17}H_{21}N_4O_2^+$ and $C_{19}H_{25}N_4O_2^+$. The MS/MS spectrum of the parent ion at m/z 341 generates characteristic peaks of the fragmentation of the pyrazolopyrimidine skeleton of sildenafil derivatives at m/z 313 and 285 ($C_{15}H_{17}N_4O_2^+$) involving two successive

losses of an ethyl group, whereas the MS/MS of the parent ion at m/z 313 produces a fragment ion at m/z 285 corresponding to the loss of only one ethyl group (Fig. 4B). Moreover, these two compounds do not have any piperazine or other amine entity (see above). All these findings are in accordance with a sildenafil-type structure in which the piperazine ring and the sulfonyl group are removed. Compound **15** is thus desulfosildenafil (DeSild) and compound **16**, DeSild with an additional ethyl group on the pyrazolopyrimidine skeleton.

Table 4 reports the measured accurate masses of the parent and fragment ions in negative ESI mode. Two characteristic ESI⁻ MS/MS spectra together with the structure of fragment ions are illustrated in Fig. S1. First, it can be noticed that compounds **3-7** and **13** are not ionized. The fragmentation of [M-H]⁻ molecular ions of methisosildenafil (**1**) and sildenafil (**12**) at m/z 487 and 473 produces ions respectively at 459 and 445 (loss of C₂H₄), 310 (loss of the piperazine-SO₂ moiety) and 282 (successive loss of these two entities). This sequential fragmentation pathway involves the neutral loss of ethylene from the ethoxyphenyl substituent and the homolytic cleavage of the C_{phenyl}-S bond already reported for sildenafil or vardenafil and their derivatives [17]. Compound **2** (MSildEt^{N26}) follows exactly the same fragmentation process confirming that the additional ethyl group compared to methisosildenafil is well located on the piperazine ring (Fig. S1). In contrast, compounds **8**, **9** and **14** only show the loss of a piperazine-SO₂ entity, which suggests that the ethoxy substituent on the benzene ring is replaced by an OH group. Thus, from the values of the molecular ion and of the eliminated piperazine-SO₂ group, it can be concluded that (i) compound **8** which undergoes the loss of a dimethylpiperazine-SO₂ entity is methisosildenafil with a hydroxyl substituent instead of an ethoxy on the benzene ring (MSildOH), (ii) compound **9** with the loss of a dimethyl-N-ethylpiperazine-SO₂ moiety is MSildOH with an additional ethyl group on the piperazine ring (MSildOHEt^{N26}), and (iii) compound **14** with the loss of a methylpiperazine-SO₂ is sildenafil with an OH instead of an ethoxy group on the benzene cycle (SildOH). MS/MS of the molecular

ions [M-H]⁻ of all the compounds, except **15**, generate a small peak at m/z 298 due to the loss of their piperazine-SO moiety (compounds **8-11** and **14**) and an additional loss of C₂H₄ (compounds **1**, **2** and **12**) (Fig. S1B and S1C) and corresponding to the molecular formula C₁₅H₁₄N₄O₃⁻ whose structure might be similar to that of the ion at m/z 299 observed in ESI⁺ mode (Fig. 4B and 4C). This is a good indication of the replacement of the ethoxy group on the benzene ring (present in compounds **1**, **2** and **12**) by a hydroxyl group in compounds **8-11** and **14**. Moreover, the detection of an ion at m/z 326 (molecular formula C₁₇H₁₈N₄O₃⁻ with a structure similar to that of the ion at m/z 327 observed in ESI⁺ mode (Fig. 4B and 4C)) for compounds **10** and **11**, indicates that they bear an ethyl group located on the pyrazolopyrimidine skeleton. In the MS spectrum of the mixture of compounds **15** and **16**, only the molecular ion [M-H]⁻ at m/z 311 of compound **15** is detected and its MS/MS fragmentation confirms the presence of an ethoxy group (loss of C₂H₄) and the absence of piperazine or other amine entity as well as of the SO₂ group.

In summary, the careful analysis of the data obtained by HRMS and HRMS/MS allows the unambiguous identification of five compounds (**2** (MSildEt^{N26}), **8** (MSildOH), **9** (MSildOHEt^{N26}), **14** (SildOH) and **15** (DeSild)). On the other hand, for the other eight compounds (**3-5**, **7**, **10**, **11**, **13** and **16**), the MS data only show that they bear an ethyl group on the pyrazolopyrimidine moiety. Compound **6** cannot be identified because its MS data lead to conflicting conclusions. An NMR study was therefore required to determine the exact structure of all these compounds and to confirm and validate the structures proposed from the MS data.

3.3.2. ¹H and ¹³C NMR analysis

The ¹H and ¹³C NMR data of all the isolated compounds are reported in Table 5. The assignments of the resonances were performed thanks to 1D and 2D (COSY, HSQC, HMBC, NOESY) NMR experiments.

¹H NMR spectra (Fig. 5) of compounds **2-11** all display, like methisosildenafil (**1**), the characteristic doublet (two for compounds **3**, **4** and **5**) of the methyl groups on the piperazine ring (CH₃ 29 and 30) between 0.99 and 1.26 ppm, thus allowing to assume they are methisosildenafil derivatives. In the same way, ¹H NMR spectra of compounds **13** and **14** exhibit, like sildenafil (**12**), at $\delta \approx 2.2$ ppm the characteristic singlet of the methyl group (CH₃ 29) linked to the N26 of the piperazine ring, thus indicating that they are sildenafil derivatives. Compared to ¹H NMR spectra of methisosildenafil or sildenafil, those of compounds **2**, **3**, **4**, **6** and **13** reveal the presence of additional characteristic resonances of one ethyl group [a triplet accounting for 3 protons (H32) and a quadruplet (or two quadruplet doublets (qd)) for 2 protons (H31)], or two ethyl groups for compounds **5** and **7** (H31/32, and H33/34). It is highly likely that these ethyl groups are positioned on the main nucleophilic sites of methisosildenafil or sildenafil derivatives, namely the oxygen and nitrogen atoms of the pyrazolo[4,3-d]pyrimidine moiety and the NH group of the piperazine moiety. The ¹H and ¹³C NMR characteristics of the CH₂ of the ethyl group should thus give a good indication of its location on the methisosildenafil or sildenafil skeleton. For compound **2**, the H31 quadruplet at 2.84 ppm shows an HMBC correlation with the C25/C27 (54.9 ppm) of the piperazine ring (Fig. 6A), thus confirming that C₂H₅ is positioned on the N26 of the piperazine moiety (MSildEt^{N26}). For compound **3**, the two H31 protons appear as two qd at 4.14 and 3.96 ppm. Although close to the quadruplet of the CH₂ (H20) of the ethoxy group on the benzene ring (4.23 ppm), the two CH₂ entities can easily be distinguished by their ¹³C resonances at 47.8 (C31) and 68.1 ppm (C20). The HMBC correlations between the qd and C5 and C9 (158.4 and 130.5 ppm) demonstrate that the N4 of the pyrazolopyrimidine moiety bears the ethyl group (Fig. 6B) (MSildEt^{N4}). For compound **4**, the CH₂ (H31) resonances show the same pattern than for compound **3** with two qd at 4.12 and 3.62 ppm for the protons and a ¹³C signal at 43.5 ppm. The HMBC correlations between CH₂ (H31) and C5 (153.1 ppm) and C7 (157.1 ppm) are the proof of the presence of the ethyl group

on the N6 of the pyrazolopyrimidine moiety (Fig. 6C) (MSildEt^{N6}). For compound **6**, the strong ¹H and ¹³C deshielding of the CH₂ (H31/C31) signals, respectively at 4.65 and 66.4 ppm, of the additional ethyl group is characteristic of an O-ethyl entity which can be distinguished from the CH₂ of the ethoxy group on the benzene ring (H20/C20) thanks to the ¹H NMR H20 δ at 4.21 ppm, the ¹³C NMR C20 δ at 67.9 ppm being non discriminant. The HMBC correlation between the CH₂ (H31) and C7 (159.1 ppm) confirms that the ethyl group is linked to the O7 of the pyrazolopyrimidine entity (Fig. 6D) (MSildEt^{O7}). Hence, a N26-ethyl substitution leads to ¹H and ¹³C CH₂ resonances respectively at ≈2.8 and 42.5 ppm. An N-ethyl location on the pyrazolopyrimidine entity gives rise to two distinct ¹H qd signals, meaning that the two CH₂ protons are inequivalent (discussed below). One of the two qd and the ¹³C resonance are more deshielded when the substitution is on N4 compared to N6 (Δδ ≈ +0.33 ppm for ¹H and +4.3 ppm for ¹³C). An O7-ethyl substitution leads to ¹H and ¹³C CH₂ signals respectively at ≈4.7 ppm and 66.5 ppm. From these considerations on the ¹H and ¹³C chemical shifts, it is possible to conclude that compound **13** is sildenafil substituted by an ethyl group on N6 (SildEt^{N6}). Regarding the two additional ethyl groups in compounds **5** and **7**, one is located on the N26 of the piperazine cycle and the other is either on the N6 (**5**) (MSildEt^{N6}Et^{N26}) or on the O7 (**7**) of the pyrazolopyrimidine skeleton (MSildEt^{O7}Et^{N26}). All these structures were unambiguously confirmed by HMBC correlations.

Ethyl signals characteristic of the O-CH₂(20)-CH₃(21) group are undetected for compounds **8-11** and **14**, demonstrating the replacement of the ethoxy group on the C19 of the benzene ring by a hydroxyl group as shown by the C19 δ (166-176 ppm) deshielded with respect to the δ of the C19 bearing an ethoxy group (163 ppm). This structural modification is also confirmed by a noticeable change in the δ of the neighboring ¹H and ¹³C resonances compared to those of methisosildenafil (**1**) or sildenafil (**12**) (shielding for H17 and H18, and deshielding for H15, C5, C18 and C19). Moreover, as no other N- or O-ethyl resonances are detected in

compounds **8** and **14**, they can be respectively identified as C19-hydroxymethisosildenafil (MSildOH) and C19-hydroxysildenafil (SildOH). Compounds **9-11**, all methisosildenafil derivatives, display respectively one ethyl group characteristic of an O7 substitution, two ethyl groups characteristic of O7 and N26 substitutions, and one ethyl group characteristic of an N26 substitution. Therefore, they are identified as MeSildOH bearing O7- (**10**) (MSildOHEt^{O7}), O7- and N26- (**11**) (MSildOHEt^{O7}Et^{N26}), and N26- (**9**) (MSildOHEt^{N26}) ethyl groups, all the structures being confirmed by HMBC correlations. The NMR spectra of the mixture of compounds **15** and **16**, compared to those of methisosildenafil or sildenafil, show for each compound the disappearance of the ¹H and ¹³C signals of the piperazine ring and the appearance of an additional ¹H triplet doublet at ≈7.1 ppm, corresponding to the presence of a hydrogen atom on the C16 of the benzene ring instead of a sulfonamide group. Therefore, the two compounds are desulfosildenafil derivatives (sildenafil without the piperazine-SO₂ moiety). Moreover, the minor compound **16** exhibits additional characteristic ¹H and ¹³C signals of an ethyl group located on the N6 of the pyrazolopyrimidine skeleton. Compound **16** is thus identified as N6-ethyl desulfosildenafil (DeSildEt^{N6}) and compound **15**, predominant in the mixture, as desulfosildenafil (DeSild). The structures of all the compounds purified from the dietary supplement are illustrated in [Fig. 7](#).

The structure of the compounds having been determined, it is possible to make a few comments on the NMR spectra, in particular on some of the proton inequivalences observed. Both protons of CH₂ 11 and CH₂ 12 of compound **3** are inequivalent leading respectively to two very close ($\Delta\nu = 1.2$ Hz) apparent triplets for CH₂ 11 and apparent sextets for CH₂ 12. This anisochrony results from the steric hindrance of the close N4-ethyl chain as shown by the nOe interactions between both CH₂ 11 and CH₂ 12 and the two protons of CH₂ 31, the interaction with H31 at 4.14 ppm being stronger than with H31 at 3.96 ppm ([Fig. S2A](#)). Confronting NMR results to calculated privileged conformers confirms that each proton of CH₂ 11 or CH₂ 12 is

closer to one of the two protons 31 than to the other (Fig. S3). Moreover, an inequivalence of the two protons of CH₂ 31 is observed in the ¹H NMR spectra of all compounds that bear an N-ethyl group on the pyrazolopyrimidine skeleton, i.e. **3**, **4**, **5**, **13** and **16**. The stronger nOe interaction between one of the two H31 (≈4.15 ppm) and aromatic H15 (≈7.8 ppm for **3**, **4**, **5**, and **13**, and 7.37 ppm for **16**) explains this finding which is confirmed by the calculated distances in the privileged conformations (Fig. S2B and S3). The shielding of the H15 signal that can be noticed for these compounds ($\Delta\delta \approx -0.44$ ppm relative to methisosildenafil H15 δ for **3**, **4**, and **5**, and -0.42 and -0.84 ppm compared to sildenafil H15 δ for **13** and **16**) (Fig. 5) could also be related to these spatial interactions.

3.3.3. Conclusive remarks

In summary, the NMR study confirms the structures of compounds **2**, **8**, **9**, **14** and **15** already established from MS data and makes it possible to unambiguously determine the position of the ethyl group on the N and/or O nucleophilic sites of the pyrazolo[4,3-d]pyrimidine backbone for compounds **3-7**, **10**, **11**, **13** and **16**, which MS does not allow. In addition, MS does not make it possible to know whether the additional ethyl group compared to methisosildenafil in compound **6** is located on the piperazine or pyrazolopyrimidine ring. Indeed, the fragmentation of its molecular ion [M+H]⁺ produces ions characteristic of the two locations, while NMR clearly shows that it is methisosildenafil substituted by an ethyl group on the O7. The presence of an ethyl group on the N26 of the piperazine as stated by the MS/MS fragmentation of the molecular ion [M+H]⁺ can be explained by an intramolecular ethyl transfer from the O7 atom of the pyrazolopyrimidine moiety to the N26 atom of the piperazine. Indeed, the methyl intramolecular transfer from the piperazine nitrogen atom to the thiocarbonyl sulfur atom of thiosildenafil derivatives is a dominant fragmentation pathway, while the methyl migration to the carbonyl oxygen atom of sildenafil may occur but to a very low extent [18].

Consequently, it can be postulated that the ethyl group on the carbonyl atom of the pyrazolopyrimidine skeleton of compound **6** can be partially transferred to the N26 of the piperazine group because when it is thus positioned, it should not migrate significantly on the carbonyl oxygen atom of methisosildenafil.

It should also be noted that identical profiles of UV spectra correspond well to molecular structures with identical chromophores (Fig. 3). The UV profile of compound **2** is similar to those of methisosildenafil (**1**) and sildenafil (**12**), indicating that, as expected, an N-ethyl substitution on the piperazine ring does not alter the chromophore. Compounds **8**, **9** and **14** with identical UV spectra are methisosildenafil or sildenafil with a hydroxyl group instead of an ethoxy group on the C19 of the benzene ring. Compared to methisosildenafil or sildenafil UV spectra, this replacement induces a bathochromic effect of ≈ 8 nm for the band at ≈ 225 nm, whereas the band at ≈ 295 nm gives rise to a vibrational fine structure between 270 and 350 nm. Compounds **4**, **5** and **13** with very similar UV spectra are N6-ethyl substituted methisosildenafil or sildenafil. The identical UV spectra of compounds **6** and **7** in one hand and **10** and **11** on the other hand are respectively characteristic of O7-ethyl substituted methisosildenafil and O7-ethyl methisosildenafil with OH instead of OC_2H_5 on the benzene ring, this change leading to bathochromic effects of ≈ 4 and 22 nm for the two main bands observed. Finally, the UV spectrum of compound **3**, a N4-ethyl substituted methisosildenafil, shows the same λ_{max} than the O7-ethyl substituted methisosildenafil derivatives (compounds **6** and **7**) but with a different profile, the most intense band of compound **3** at 205.7 nm appearing as a shoulder for compounds **6** and **7** at 210 nm.

3.4. Quantitative analysis of methisosildenafil, sildenafil, and related compounds in the dietary supplement analyzed

The quantities of methisosildenafil (**1**), sildenafil (**12**), and compounds (**8**) (MSildOH) and (**4**) (MSildEt^{N26}) were easily measured from the signal areas of their aromatic protons (Fig. S4) in the conditions described in reference [1]. They account respectively for 38, 20, 5 and 3 mg per capsule, i.e. 54%, 32%, 7% and 4% of the total contaminants. The amounts of the other products could only be estimated due to the very low intensity of their ¹H NMR signals (except for MSildEt^{N26} (**2**) which has no distinctive resonances); they range from 0.01 to 0.50 mg per capsule and represent ≈3% of the total contaminants, i.e. ≈2 mg per capsule. So, each capsule contains ≈68 mg of methisosildenafil- and sildenafil-related compounds, i.e. between the recommended (50 mg) and the maximum (100 mg) daily dose of sildenafil in patients.

4. Discussion

This study deals with the purification and characterization of 16 sildenafil derivatives present in a dietary supplement intended to be commercialized for improving sexual performance and alleged to be “100% natural”. Two adulterants are found in large quantities: sildenafil, the lead PDE-5i drug, and methisosildenafil, a sildenafil analogue that has never received a marketing authorization as a medicine. All other molecules possess the scaffold of sildenafil, i.e. the 5-phenyl-pyrazolo[4,3-d]pyrimidine moiety, and twelve out of fourteen bear a sulfonyl group linked to a piperazine ring (Fig. 7). Their structures mainly differ by the presence or absence of an ethyl group N- or O- linked to either the pyrazolopyrimidine or piperazine moiety. Besides sildenafil commonly found in adulterated erectile dietary supplements, five other compounds have already been described in the literature. Methisosildenafil was first reported in 2007 as an adulterant of an herbal dietary supplement [19] and MSildEt^{N26} (**2**) in 2018 in health foods analyzed by UHPLC coupled with Q-TOF-MS [20]. DeSild (**15**), also named imidazosagatriazinone, is known as sildenafil lactam impurity and is commercially available (CAS number 139756-21-1). However, it has never been reported

in a dietary supplement. SildOH (**14**) is the Sildenafil Impurity C in the European Pharmacopoeia and is also commercially available (CAS number 139755-91-2). SildEt^{N6} (**13**) was described as an impurity formed during the synthesis of sildenafil but was not thoroughly characterized [21]. The 10 other chemicals identified in this study have to our knowledge never been described in the literature.

The presence of numerous sildenafil derivatives in the dietary supplement analyzed is most probably due to its intentional adulteration with sildenafil and methisosildenafil. It can be hypothesized that the minor compounds detected are either degradation impurities ensuing from the effect of humidity and/or light during storage for instance, or by-products of different reaction steps during the synthesis of these two PDE-5i that have not been properly eliminated during the purification procedure. The second hypothesis is preferred because neither the hydrolysis nor the photodegradation of sildenafil and analogues leads to the kind of structures identified in the present study [19,22]. In order to correlate the chemical structures of the minor components identified in the dietary supplement with the different synthetic routes to sildenafil and methisosildenafil, we thus thoroughly analyzed the reaction sequences towards both APIs from the literature [23,24]. The preparation of sildenafil and methisosildenafil follows the same overall approach, except for the piperazine used, that is either N-methylated or 2,6-dimethylated, respectively. Two main types of synthetic routes can be distinguished, the first-generation ones relying on the late introduction of the sulfonamide moiety and the more recent ones based on an early sulfonylation step.

According to the chemical structures assigned to the minor compounds detected, one can subdivide the discussion into two main categories, illustrated for sildenafil in Fig. 8. The first one deals with compounds **15** and **16**, lacking the sulfonamide group. The second one comprises all the other compounds that display either no ethyl radical (compounds **8** and **14**) or variable O- or N-ethylation patterns (**2-7**, **9-11**, **13**). It has to be recalled that, in the case of

methisosildenafil, the presence of the free piperazine nitrogen offers an additional opportunity for unwanted ethylation.

The presence of unsulfonylated compounds can be explained by at least two principal reaction sequences. The initial medicinal chemistry route described for sildenafil involves a late sulfonylation step [25], which is known to be reversible and thus potentially leads to desulfonylated by-products [26] (Fig. 8, pathway A). As an alternative, a late Friedel-Crafts process with sulfamoyl chloride in the presence of AlCl₃ was proposed to introduce the sulfonamide moiety (Fig. 8, pathway B) [23,25]. This procedure was described with only 45% yield, potentially leaving a large amount of starting reagent in the reaction medium. A neglected purification step would thus explain the presence of unsulfonylated by-products in both cases.

The occurrence of variable O- or N-ethylation patterns, corresponding to the second class of by-products, can be correlated with at least three different synthetic pathways. In the first possible pathway, the late sulfonylation step requires the *para*-chlorosulfonylation of an ethoxyphenyl intermediate with chlorosulfonic acid (Fig. 8, pathway C). The scaling-up of this reaction was reported to be troublesome because of the increased quench time of the excess of chlorosulfonic acid that leads to prolonged exposure to a large amount of hydrochloric acid [23]. The possible hydrolysis of the O-ethoxy group would explain the presence of a free phenol in compounds **8**, **10** and **14**, as previously proposed in a study on synthetic impurities of sildenafil [27]. Such conditions could in turn result in the formation of ethyl chloride susceptible to alkylate the electron-rich positions of the pyrimidinone core of the molecule. Alternatively, the highly acidic medium could also potentially induce a direct intra- or intermolecular nucleophilic transfer of the ethoxy radical. Yet, due to the absence of nucleophilicity of the protonated methisosildenafil piperazine ring nitrogen, the formation of compounds **2**, **5**, **7**, **9** and **11** is unlikely under these acidic conditions. The observed random distribution of ethyl radicals rather suggests side-products arising from neutral or basic reaction media.

More recent synthetic pathways of sildenafil were reported involving an early introduction of the sulfonamide moiety so as to circumvent the problems associated with the late-stage transformation. Two distinct synthetic steps that share the use of basic conditions can be proposed in this case. The first one relies on a critical intramolecular cyclization reaction, using for instance *t*-BuOK in *t*-BuOH, to generate the pyrazolopyrimidone core of sildenafil (Fig. 8, pathway D). The ethoxy moiety present in the benzamide precursor was described to be doubly activated by the sulfonamide and amide groups, and thus prone to intra- or intermolecular nucleophilic substitution [23,24]. This transformation would lead to either the loss of the ethyl radical or its transfer onto the different nucleophilic sites of the molecule. Another synthetic pathway was then proposed to address this issue, in which the whole synthesis is run with a free phenol group and the O-alkylation of the phenol is postponed after the cyclisation reaction [21] (Fig. 8, pathway E). Unless a very specific neutral decarboxylative alkylation of a carbonate intermediate was used, exposure to standard alkylating agents was described to lead to pyrimidinone O- or N-ethylation, along with partial phenol alkylation, eventually giving rise to a product distribution similar to that observed in the present study.

Overall, the analysis of the reported routes towards sildenafil and methisosildenafil indicates that all the minor compounds identified in the dietary supplement can be side-products of relevant synthetic steps. It was not possible to unambiguously decipher the synthetic sequence used, or even if there was only one. This clearly indicates that the synthesis processes used by the manufacturers do not meet the standard chemical purification requirements. A similar problematic of erectile dietary supplement containing synthetic impurities in minor amounts was previously reported in two articles by Schramek et al. In the first study [28], the dietary supplement contained dithiodesmethylcarbodenafil and three impurities coming from its synthetic pathway and in the second study [29], the herbal food supplement contained 14 chemicals (5 never described) bearing the basic structure of sildenafil that might be process-

related impurities or by-products of PDE-5i analogues synthesis. So, in addition to the illegal falsification of the food supplement by PDE-5i, our study shows once again that manufacturers do not respect good manufacturing practices and do not control the quality of their preparation before sending it to intermediaries for placing on the market.

It must be underlined that there is a real health risk for a consumer who would ingest the dietary supplement analyzed. First, it contains a non-negligible amount of sildenafil (20 mg per capsule) which corresponds to about half of the recommended therapeutic daily dose. Second, it contains methisosildenafil (38 mg per capsule) and other analogues (10 mg per capsule) whose safety and toxicology profiles are unknown as they have not been subjected to any clinical trials. Being structurally similar to sildenafil, these analogues may retain the corresponding pharmacological/toxicological properties or have different properties, thus leading to unpredictable effects and/or side-effects [3]. For example, a fatal case associated with the ingestion of a sildenafil analogue (desmethylcarbodenafil) has been reported in 2017 [30]. In the case of the dietary supplement described in this study, beyond the risks posed by each compound present in the commercial preparation, the possibility of a synergistic effect of the 16 different molecules must also be taken into account.

5. Conclusion

The “all-natural” dietary supplement for enhancing sexual performance investigated in this study contains a mixture of two major PDE-5i adulterants, sildenafil and methisosildenafil, intentionally added to increase its effect and fourteen minor compounds. All compounds were isolated thanks to the orthogonality of SFC and LC separations and their structures elucidated using UV spectroscopy, high resolution and tandem mass spectrometry and one- and two-dimensional NMR. The minor contaminants come from poor purification procedures during the

different steps of sildenafil and methisosildenafil synthesis and 10 of them have never been described in the literature.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:

Figure S1. (A) HRMS/MS spectra in negative ESI mode of two methisosildenafil-related compounds (**2**, **11**) purified from the dietary supplement analyzed. The formulae shown represent the molecules and not the ions. Proposed structures of fragment ions of (B) compounds with an ethoxy group on the benzene ring, (C) compounds with a hydroxyl group on the benzene ring.

Figure S2. Parts of 2D NOESY spectra showing the spatial correlations between CH₂ 11, 12 and H31 for compound **3** and between H15 and CH₂ 31 for compound **4**.

Figure S3. Representation of a privileged conformation calculated for compound **3** and interatomic distances between protons of interest.

Figure S4. Part (6.7-8.9 ppm) of the ^1H NMR spectrum with ^{13}C GARP broadband decoupling of the dietary supplement recorded in $\text{CD}_3\text{CN}/\text{D}_2\text{O}$ (80:20, v/v) with attribution of the signals of the identified compounds.

Captions for figures

Figure 1. (A) SFC-UV chromatogram on BetaSil Diol-100 column (the collection windows are indicated by the vertical dashed lines). (B) Preparative LC-UV chromatograms on C18 reverse phase columns. All experimental conditions are reported in [Table 1](#). The presence of compounds annotated 12* and 1* in W2 and W3 fractions may be due to the high concentration of compounds **12** and **1** in the C2 fraction.

Figure 2. ^1H NMR spectrum with ^{13}C GARP broadband decoupling of the dietary supplement recorded in $\text{CD}_3\text{CN}/\text{D}_2\text{O}$ (80:20, v/v). (A), (B), (C) and (D) correspond to zoomed areas highlighted by boxes on the full spectrum. The main signals of sildenafil (**12**) and methisosildenafil (**1**) are shown.

Figure 3. UV spectra of methisosildenafil (MSild), sildenafil (Sild) and their related compounds purified from the dietary supplement analyzed. Spectra were obtained from the UHPLC analysis of the purified fractions.

Figure 4. (A) HRMS/MS spectra in positive ESI mode of methisosildenafil (MSild, **1**), sildenafil (Sild, **12**) and related compounds (**2**, **4**, **11** and **13**) purified from the dietary supplement analyzed. The formulae shown represent the molecules and not the ions. Proposed structures of fragment ions of (B) compounds with an ethoxy group on the benzene ring, (C) compounds with a hydroxyl group on the benzene ring, (D) piperazine entities.

Figure 5. ^1H NMR spectra of methisosildenafil (MSild), sildenafil (Sild), and related compounds purified from the dietary supplement analyzed. Spectra were recorded in

CD₃CN/D₂O (80:20, v/v) except for compounds **9** and **14** whose solubilization required the subsequent addition of 5 µL of 0.5 M NaOD in the NMR tube. Regions 6.7-9.0 ppm (A), 2.5-5.0 ppm (B), 1.6-2.6 ppm (C) and 0.7-1.6 ppm (D). The intensity scale is different for the different parts of the spectrum. For compounds **15** and **16** that could not be separated, the prime symbol (') is used to mark the protons of compound **16**. HOD: residual protons in D₂O; CD₂HCN: residual protons in CD₃CN; S_{CD₂HCN}: ¹³C satellite signals of CD₂HCN; ax: axial protons; eq: equatorial protons; FA: fatty acids present in the dietary supplement; MeOH: methanol; Ac: residual acetate from the chromatographic mobile phase; *: unknown contaminants.

Figure 6. Parts of the ¹H-¹³C HMBC spectra of compounds **2** (A), **3** (B), **4** (C) and **6** (D). Correlations between protons H31 and specific carbons indicating the position of the ethyl group in the structure are marked with dashed arrows. See Fig. 7 for chemical structures and numbering. MeOH: methanol; HOD: residual protons in D₂O.

Figure 7. Chemical structures of sildenafil, methisosildenafil and their analogues purified and characterized in this study. (a) CAS registry number; (b) compound already described in reference [20]; (c) compound already described in reference [21]; (d) known as Sildenafil European Pharmacopoeia impurity C; (e) known as imidazosagatriazinone.

Figure 8. Possible origin of the various impurities found in the food supplement analyzed, according to the main routes of synthesis of sildenafil and methisosildenafil reported in the literature [21,23-27]. Pip: N-methylpiperazine or 2,6-dimethylpiperazine.

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Figures 1-8
(A) Figure 1

Figure 2

Figure 3

(A) **Figure 4**

(B)

m/z
432 460

m/z 166
Common to all
compounds

m/z without ethyl group on the pyrazolopyrimidine moiety

Pip A: 2,6-dimethylpiperazine 489

Pip B: N1-ethyl-2,6-dimethylpiperazine 517

Pip C: N-methylpiperazine 475

m/z with ethyl group

517

545

503

377

405

m/z
299 327

m/z 311 339

312 340

313 341

m/z 283 311

285 313

(C)

m/z without ethyl group on the pyrazolopyrimidine moiety

Pip A: 2,6-dimethylpiperazine 461

Pip B: N1-ethyl-2,6-dimethylpiperazine 489

Pip C: N-methylpiperazine 447

m/z with ethyl group

489

517

347

375

m/z 283 311

299 327

(D)

- 2,6-dimethylpiperazine

m/z 113
 $C_6H_{13}N_2^+$

99
 $C_5H_{11}N_2^+$

84.0813
 $C_5H_{10}N^+$

71
 $C_4H_9N^{\bullet+}$

- N1-ethyl-2,6-dimethylpiperazine

m/z 141
 $C_8H_{17}N_2^+$

127
 $C_7H_{15}N_2^+$

113
 $C_6H_{13}N_2^+$

100.1126
 $C_6H_{14}N^+$

99
 $C_5H_{11}N_2^+$

and

86
 $C_5H_{12}N^+$

84.0813
 $C_5H_{10}N^+$

and

72
 $C_4H_{10}N^+$

71
 $C_4H_9N^{\bullet+}$

- N1-methylpiperazine

m/z 163
 $C_5H_{11}N_2O_2S^+$

100.1000
 $C_5H_{12}N_2^{\bullet+}$

99
 $C_5H_{11}N_2^+$

84.0688
 $C_4H_8N_2^{\bullet+}$

70
 $C_4H_8N^+$

58
 $C_3H_8N^+$

The two ions at m/z 70 and 58 are also observed with 2,6-dimethylpiperazine and N1-ethyl-2,6-dimethylpiperazine.

Figure 5

Figure 5

Figure 5

Figure 5
(C)

(D)

Figure 6

Figure 7

(1) MSild
Methisosildenafil
[496835-35-9]^(a)

(2) MSildEt^{N26}
N26-ethyl-methisosildenafil^(b)

(3) MSildEt^{N4}
N4-ethyl-methisosildenafil

(4) MSildEt^{N6}
N6-ethyl-methisosildenafil

(5) MSildEt^{N6}Et^{N26}
N6,N26-diethyl-methisosildenafil

(6) MSildEt⁰⁷
07-ethyl-methisosildenafil

(7) MSildEt^{O7}Et^{N26}
O7,N26-diethyl-methisosildenafil

(8) MSildOH
O-desethyl-methisosildenafil

(9) MSildOHEt^{N26}
N26-ethyl-O-desethyl-
methisosildenafil

(10) MSildOHEt⁰⁷
O7-ethyl-O-desethyl-
methisosildenafil

(11) MSildOHEt⁰⁷Et^{N26}
07,N26-diethyl-O-desethyl-
methisosildenafil

(12) Sild
Sildenafil
 [139755-83-2] (a)

(13) SildEt^{N6}
N6-ethyl-sildenafil (c)

(14) SildOH
O-desethyl-sildenafil
 [139755-91-2] (a,d)

(15) DeSild
Desulfosildenafil
 [139756-21-1] (a,e)

(16) DeSildEt^{N6}
N6-ethyl-desulfosildenafil

Figure 8

Sildenafil ($R_1 = \text{Me}$, $R_2 = \text{H}$)
Methisosildenafil ($R_1 = \text{H}$, $R_2 = \text{Me}$)

Unsulfonylated impurities

Randomly ethylated impurities

(A)

Chlorosulfonylation

(B)

Friedel-Crafts

Reversible transformation Incomplete conversion

15,16

Late sulfonylation

Early sulfonylation

(C)

Chlorosulfonylation

Nucleophilic transfer under
acidic conditions

3, 4, 6, 8, 10, 13, 14, 16

Cyclisation

(D)

Nucleophilic transfer under
basic conditions

2-11, 13, 14, 16

Alkylation

(E)

Unselective
alkylation

Author statement

Gaëtan Assemat: Methodology, Validation, Investigation, Writing-original draft preparation, Visualization. **Stéphane Balayssac:** Methodology, Validation, Investigation, Visualization, Supervision. **Véronique Gilard:** Validation, Writing-original draft preparation, Visualization, Supervision, Funding acquisition. **Nathalie-Martins-Froment:** Investigation. **Isabelle Fabing:** Investigation. **Frédéric Rodriguez:** Formal analysis. **Yves Génisson:** Writing-review and editing. **Robert Martino:** Writing-review and editing. **Myriam Malet-Martino:** Conceptualization, Writing-review and editing, Supervision, Funding acquisition.

Table 1. Chromatographic conditions for purification of sildenafil and its 15 analogues from the dietary supplement analyzed.

		SFC conditions ^a		Preparative LC conditions ^a								Analytical UHPLC ^a	
Fraction used		Raw		A	B	C	D	E	F	G		All	
Instrument		Berger Multigram II preparative SFC		Waters Prep 150 LC	Waters Prep 150 LC	Waters Prep 150 LC		Waters Prep 150 LC				Waters Acquity UHPLC	
Column		BetaSil Diol-100 ^b		Kromasil C18 ^c	CSH C18 ^c	Kromasil C18 ^c		Kromasil C18 ^c				Acquity UHPLC CSH C18 ^d	
Mobile phase	(A)	CO ₂ (100 bar)		10 mM ammonium acetate	0.1% (v/v) TFA ^e in water	10 mM ammonium acetate		10 mM ammonium acetate				0.1% (v/v) TFA ^e in water	
	(B)	0.5% (v/v) isopropylamine in methanol		10% (v/v) of 100 mM ammonium acetate in acetonitrile	10% (v/v) of mobile phase (A) in acetonitrile	10% (v/v) of 100 mM ammonium acetate in acetonitrile		10% (v/v) of 100 mM ammonium acetate in acetonitrile				0.1% (v/v) TFA ^e in acetonitrile	
Gradient elution profile	Step	min	% (B)	min	% (B)	min	% (B)	min	% (B)	min	% (B)	min	% (B)
	1	0 - 0.5	10	0 - 50	30 - 100	0 - 40	10 - 70	0 - 40	30 - 70	0 - 40	25 - 70	0 - 4	5 - 95
	2	0.5 - 8	10 - 28	50 - 55	100	40 - 41	70 - 100	40 - 41	70 - 100	40 - 41	70 - 100	4 - 5.5	95
	3	8 - 15	28			41 - 50	100	41 - 50	100	41 - 50	100	5.5 - 5.51	5
	4	15 - 16	28 - 10									5.51 - 7.3	5
	5	16 - 20	10										
Flow rate		42.5 mL/min		120 mL/min		120 mL/min		120 mL/min		120 mL/min		0.6 mL/min	
Temperature		40 °C		RT		RT		RT		RT		30 °C	
Injection volume ^f		0.5 mL		9 mL		7 mL		40 mL	10 mL	10 mL	12 mL	8 mL	1 µL
Compounds collected		All		13,2,5,15,16,10,7,11		4,6		1,12	3	9	14	8	

^a UV detection at 235 nm and for analytical UHPLC, cone voltage of QDa mass detector used in positive mode set at 30 V.

^b 250 mm × 21.2 mm i.d., 5 µm particle size.

^c 250 mm × 50 mm i.d., 10 µm particle size.

^d 100 mm × 2.1 mm i.d., 1.7 µm particle size.

^e TFA: trifluoroacetic acid.

^f The concentration of powder dissolved in each fraction is different: 50 mg/mL for SFC, 6.3 mg/mL for A, 8.5 mg/mL for B, 4.8 mg/mL for C, 18.6 mg/mL for D, 8.5 mg/mL for E, 6.8 mg/mL for F and 18.5 mg/mL for G.

Table 2. Retention times and masses of the parent ions ($[M+H]^+$) of purified compounds in UHPLC.

Compound number	Retention time ^a (min)	UHPLC-MS $[M+H]^+$
1	2.15	489
2	2.21	517
3	2.09	517
4	2.25	517
5	2.31	545
6	2.58	517
7	2.64	545
8	2.22	461
9	2.27	489
10	2.86	489
11	2.92	517
12	2.07	475
13	2.16	503
14	2.15	447
15	3.42	313
16	3.42	341

^a UHPLC analytical conditions for the control of the chemical purity of preparative LC samples are reported in [Table 1](#).

Table 3. Accurate mass data of parent and fragment ions in positive ESI mode for all the compounds (Cpd) isolated from the dietary supplement analyzed. Mass of parent ion is indicated in bold.

A: methisosildenafil (MSild) and compounds 2-11

Theoretical mass and formula	Experimental mass of parent and fragment ions (relative mass error in ppm)										
	MSild(1)	Cpd (2)	Cpd (3)	Cpd (4)	Cpd (5)	Cpd (6)	Cpd (7)	Cpd (8)	Cpd (9)	Cpd (10)	Cpd (11)
545.2910					545.2920		545.2922				
C ₂₇ H ₄₁ N ₆ O ₄ S ⁺					(+1.8)		(+2.2)				
517.2597		517.2599	517.2595	517.2587	517.2593	517.2591	517.2587				517.2600
C ₂₅ H ₃₇ N ₆ O ₄ S ⁺		(+0.4)	(-0.4)	(-1.9)	(-0.8)	(-1.2)	(-1.9)				(+0.6)
489.2284	489.2277	489.2277	489.2278	489.2273	489.2272	489.2276	489.2271		489.2292	489.2292	
C ₂₃ H ₃₃ N ₆ O ₄ S ⁺	(-1.4)	(-1.4)	(-1.2)	(-2.2)	(-2.5)	(-1.6)	(-2.7)		(+1.6)	(+1.6)	
461.1971								461.1973			
C ₂₁ H ₂₉ N ₆ O ₄ S ⁺								(+0.4)			
460.2019			460.2007	460.2015	460.2011	460.2008	460.2008				
C ₂₂ H ₃₀ N ₅ O ₄ S ⁺			(-2.6)	(-0.9)	(-1.7)	(-2.4)	(-2.4)				
432.1700	432.1692	432.1696	432.1690	432.1696	432.1694	432.1695	432.1690				
C ₂₀ H ₂₆ N ₅ O ₄ S ⁺	(-1.9)	(-0.9)	(-2.3)	(-0.9)	(-1.4)	(-1.2)	(-2.3)				
405.1597			405.1592	405.1588	405.1591	405.1591	405.1588				
C ₁₉ H ₂₅ N ₄ O ₄ S ⁺			(-1.2)	(-2.2)	(-1.5)	(-1.5)	(-2.2)				
377.1278	377.1273	377.1272	377.1281	377.1275	377.1273	377.1277	377.1274				
C ₁₇ H ₂₁ N ₄ O ₄ S ⁺	(-1.3)	(-1.6)	(+0.8)	(-0.8)	(-1.3)	(-0.3)	(-1.1)				
375.1127										375.1127	375.1125
C ₁₇ H ₁₉ N ₄ O ₄ S ⁺										(0)	(-0.5)
347.0814								347.0812	347.0815		
C ₁₅ H ₁₅ N ₄ O ₄ S ⁺								(-0.6)	(+0.3)		
340.1899			340.1895	340.1897							
C ₁₉ H ₂₄ N ₄ O ₂ ⁺			(-1.2)	(-0.6)							
339.1815				339.1816	339.1815	339.1815	339.1817				
C ₁₉ H ₂₃ N ₄ O ₂ ⁺				(+0.3)	(0)	(0)	(+0.6)				
327.1458			327.1464	327.1450	327.1445	327.1446	327.1472			327.1458	327.1453
C ₁₇ N ₁₉ N ₄ O ₃ ⁺			(+1.8)	(-2.4)	(-4.0)	(-3.7)	(+4.3)			(0)	(-1.5)
312.1580			312.1575	312.1584							
C ₁₇ H ₂₀ N ₄ O ₂ ⁺			(-1.6)	(+1.3)							

311.1502 C ₁₇ H ₁₉ N ₄ O ₂ ⁺	311.1503 (+0.3)	311.1506 (+1.3)	311.1508 (+1.9)	311.1504 (+0.6)	311.1501 (-0.3)	311.1505 (+1.0)	311.1500 (+0.6)			311.1510 (+2.6)	311.1503 (+0.3)
299.1144 C ₁₅ H ₁₅ N ₄ O ₃ ⁺	299.1142 (-0.7)	299.1141 (-1.0)	299.1137 (-2.3)	299.1139 (-1.6)	299.1145 (+0.3)	299.1141 (-1.0)	299.1138 (-2.0)	299.1147 (+1.0)	299.1147 (+1.0)	299.1146 (+0.7)	299.1143 (-0.3)
284.1268 C ₁₅ H ₁₆ N ₄ O ₂ ⁺			284.1272 (+1.4)	284.1269 (+0.4)					284.1266 (-0.7)		
283.1190 C ₁₅ H ₁₅ N ₄ O ₂ ⁺	283.1190 (0)	283.1195 (+1.8)	283.1195 (+1.8)	283.1191 (+0.4)	283.1188 (-0.7)	283.1192 (+0.7)	283.1190 (0)	283.1198 (+2.8)	283.1196 (+2.1)	283.1194 (+1.4)	283.1189 (-0.4)
166.0980 C ₈ H ₁₂ N ₃ O ⁺	166.0979 (-0.6)	166.0982 (+1.2)	166.0985 (+3.0)	166.0978 (-1.2)	166.0984 (+2.4)	166.0981 (+0.6)	166.0973 (-4.2)	166.0981 (+0.6)	166.0980 (0)	166.0981 (+0.6)	166.0973 (-4.2)
143.1548 C ₈ H ₁₉ N ₂ ⁺									143.1551 (+2.1)		
141.1392 C ₈ H ₁₇ N ₂ ⁺		141.1394 (+1.4)	141.1392 (0)	141.1396 (+2.8)	141.1392 (0)	141.1390 (-1.4)	141.1391 (-0.7)		141.1392 (0)		141.1392 (0)
127.1235 C ₇ H ₁₅ N ₂ ⁺		127.1238 (+2.4)	127.1237 (+1.6)	127.1239 (+3.1)	127.1236 (+0.8)	127.1234 (-0.8)	127.1236 (+0.8)		127.1235 (0)		127.1233 (-1.6)
115.1230 C ₆ H ₁₅ N ₂ ⁺								115.1235 (+4.3)			
113.1079 C ₆ H ₁₃ N ₂ ⁺	113.1077 (-1.8)	113.1081 (+1.8)	113.1077 (-1.8)	113.1076 (-2.7)	113.1079 (0)	113.1081 (+1.8)	113.1081 (+1.8)	113.1080 (+0.9)	113.1084 (+4.4)	113.1079 (0)	113.1076 (-2.7)
100.1126 C ₆ H ₁₄ N ⁺		100.1130 (+4.0)			100.1127 (+1.0)	100.1134 (+8.0)	100.1129 (+3.0)		100.1127 (+1.0)		100.1127 (+1.0)
99.0922 C ₅ H ₁₁ N ₂ ⁺	99.0923 (+1.0)		99.0928 (+6.1)	99.0921 (-1.0)		99.0927 (+5.0)		99.0924 (+2.0)		99.0925 (+3.0)	
98.0970 C ₆ H ₁₂ N ⁺		98.0972 (+2.0)			98.0972 (+2.0)	98.0969 (-1.0)	98.0972 (+2.0)		98.0971 (+1.0)		98.0971 (+1.0)
86.0970 C ₅ H ₁₂ N ⁺		86.0973 (+3.5)			86.0972 (+2.3)	86.0976 (+7.0)	86.0973 (+3.5)		86.0976 (+7.0)		86.0972 (+2.3)
84.0813 C ₅ H ₁₀ N ⁺	84.0814 (+1.2)	84.0818 (+5.9)	84.0821 (+9.5)	84.0820 (+8.3)	84.0816 (+3.6)	84.0816 (+3.6)	84.0813 (0)	84.0819 (+7.1)	84.0816 (+3.6)	84.0816 (+3.6)	84.0807 (-7.1)
72.0813 C ₄ H ₁₀ N ⁺		72.0818 (+6.9)			72.0816 (+4.2)	72.0814 (+1.4)	72.0812 (-1.4)		72.0815 (+2.8)		72.0816 (+4.2)
71.0735 C ₄ H ₉ N ⁺ •	71.0739 (+5.6)	71.0741 (+8.4)	71.0737 (+2.8)	71.0738 (+4.2)	71.0740 (+7.0)	71.0741 (+8.4)	71.0741 (+8.4)	71.0738 (+4.2)	71.0741 (+8.4)	71.0741 (+8.4)	71.0740 (+7.0)

B: sildenafil (Sild) and compounds 13-16

Theoretical mass and formula	Experimental mass of parent and fragment ions (relative mass error in ppm)				
	Sild (12)	Cpd (13)	Cpd (14)	Cpd (15)	Cpd (16)
503.2440		503.2448			
C ₂₄ H ₃₅ N ₆ O ₄ S ⁺		(+1.6)			
475.2127	475.2126	475.2119			
C ₂₂ H ₃₁ N ₆ O ₄ S ⁺	(-0.2)	(-1.7)			
447.1815			447.1818		
C ₂₀ H ₂₇ N ₆ O ₄ S ⁺			(+0.7)		
405.1597		405.1596			
C ₁₉ H ₂₅ N ₄ O ₄ S ⁺		(-0.2)			
377.1278	377.1270	377.1296			
C ₁₇ H ₂₁ N ₄ O ₄ S ⁺	(-2.1)	(+4.8)			
347.0814			347.0809		
C ₁₅ H ₁₅ N ₄ O ₄ S ⁺			(+1.4)		
341.1978					341.1980
C ₁₉ H ₂₅ N ₄ O ₂ ⁺					(+0.6)
340.1899		340.1883			
C ₁₉ H ₂₄ N ₄ O ₂ ⁺		(-4.7)			
339.1815		339.1816			
C ₁₉ H ₂₃ N ₄ O ₂ ⁺		(+0.3)			
327.1458		327.1465			
C ₁₇ H ₁₉ N ₄ O ₃ ⁺		(+2.1)			
313.1658				313.1663	313.1666
C ₁₇ H ₂₁ N ₄ O ₂ ⁺				(+1.6)	(+2.6)
311.1502	311.1504	311.1510			
C ₁₇ H ₁₉ N ₄ O ₂ ⁺	(+0.6)	(+2.6)			
299.1144	299.1140	299.1151	299.1148		
C ₁₅ H ₁₅ N ₄ O ₃ ⁺	(-1.3)	(+2.3)	(+1.3)		
285.1346				285.1352	285.1356
C ₁₅ H ₁₇ N ₄ O ₂ ⁺				(+2.1)	(+3.5)
283.1190	283.1192	283.1194	283.1198		
C ₁₅ H ₁₅ N ₄ O ₂ ⁺	(+0.7)	(+1.4)	(+2.8)		

166.0980	166.0979	166.0983	166.0981	166.0981	166.0980
C ₈ H ₁₂ N ₃ O ⁺	(-0.6)	(+1.8)	(+0.6)	(+0.6)	(0)
163.0541	163.0539	163.0543	163.0549		
C ₅ H ₁₁ N ₂ O ₂ S ⁺	(-1.2)	(+1.2)	(+4.9)		
101.1079			101.1081		
C ₅ H ₁₃ N ₂ ⁺			(+2.0)		
100.1000	100.1001	100.1001	100.1001		
C ₅ H ₁₂ N ₂ ⁺ •	(+1.0)	(+1.0)	(+1.0)		
99.0922	99.0923	99.0924	99.0925		
C ₅ H ₁₁ N ₂ ⁺	(+1.0)	(+2.0)	(+3.0)		
84.0688	84.0691	84.0688			
C ₄ H ₈ N ₂ ⁺ •	(+3.6)	(0)			
70.0657 ^a	70.0662	70.0662	70.0658		
C ₄ H ₈ N ⁺	(+7.1)	(+7.1)	(+1.4)		
58.0661 ^a	58.0664	58.0662	58.0661		
C ₃ H ₈ N ⁺	(+5.2)	(+1.7)	(0)		

^a The ions at m/z 70 and 58 are also observed in the degradation pathways of methisosildenafil (MSild) and compounds **2-11** although not mentioned in [Table 3A](#).

Table 4. Accurate mass data of parent and fragment ions in negative ESI mode for sildenafil (Sild) and some of its analogues found in the dietary supplement analyzed. Mass of parent ion is indicated in bold. Cpd: compound; MSild: methisosildenafil.

Theoretical mass	Formula	Experimental mass (relative mass error in ppm)								
		MSild (1)	Cpd (2)	Cpd (8)	Cpd (9)	Cpd (10)	Cpd (11)	Sild (12)	Cpd (14)	Cpd (15)
515.2440	C ₂₅ H ₃₅ N ₆ O ₄ S ⁻		515.2448 (+1.6)				515.2442 (+0.4)			
487.2127	C ₂₃ H ₃₁ N ₆ O ₄ S ⁻	487.2133 (+1.2)	487.2124 (-0.6)		487.2130 (+0.6)	487.2144 (+3.5)	487.2124 (-0.6)			
473.1971	C ₂₂ H ₂₉ N ₆ O ₄ S ⁻							473.1979 (+1.7)		
459.1814	C ₂₁ H ₂₇ N ₆ O ₄ S ⁻	459.1810 (-0.9)		459.1813 (-0.2)		459.1817 (+0.7)				
445.1658	C ₂₀ H ₂₅ N ₆ O ₄ S ⁻							445.1652 (-1.3)	445.1653 (-1.1)	
326.1379	C ₁₇ H ₁₈ N ₄ O ₃ ⁻					326.1386 (+2.1)	326.1373 (-1.8)			
311.1508	C ₁₇ H ₁₉ N ₄ O ₂ ⁻									311.1509 (+0.3)
310.1430	C ₁₇ H ₁₈ N ₄ O ₂ [•]	310.1427 (-1.0)	310.1422 (-2.6)			310.1431 (+0.3)	310.1426 (-1.3)	310.1436 (+1.9)		
298.1066	C ₁₅ H ₁₄ N ₄ O ₃ ⁻	298.1073 (+2.3)	298.1074 (+2.7)	298.1062 (-1.3)	298.1072 (+2.0)	298.1066 (0)	298.1075 (+3.0)	298.1062 (-1.3)	298.1071 (+1.7)	
283.1195	C ₁₅ H ₁₅ N ₄ O ₂ ⁻									283.1192 (-1.1)
282.1117	C ₁₅ H ₁₄ N ₄ O ₂ [•]	282.1114 (-1.1)	282.1114 (-1.1)	282.1117 (0)	282.1119 (+0.7)	282.1112 (-1.8)	282.1116 (-0.4)	282.1115 (-0.7)	282.1114 (-1.1)	

Table 5. ^1H and ^{13}C NMR data of methisosildenafil (MSild), sildenafil (Sild) and their related compounds purified from the dietary supplement analyzed. δ were measured in $\text{CD}_3\text{CN}:\text{D}_2\text{O}$ (80:20).

	MSild (1)				MSildEt ^{N4} (3)				MSildEt ^{N6} (4)				MSildEt ^{O7} (6)

N ^o (a)	δ ^1H (ppm)	Multiplicity ^(b) (J, Hz)		δ ^{13}C (ppm)	δ ^1H (ppm)	Multiplicity ^(b) (J, Hz)		δ ^{13}C (ppm)	δ ^1H (ppm)	Multiplicity ^(b) (J, Hz)		δ ^{13}C (ppm)	δ ^1H (ppm)	Multiplicity ^(b) (J, Hz)		δ ^{13}C (ppm)
3	-	-	-	148.9	-	-	-	140.7	-	-	-	148.2	-	-	-	148.3
5	-	-	-	150.7	-	-	-	158.4	-	-	-	153.1	-	-	-	158.4
7	-	-	-	157.0	-	-	-	164.7	-	-	-	157.1	-	-	-	159.1
8	-	-	-	127.4	-	-	-	129.4	-	-	-	127.5	-	-	-	124.2
9	-	-	-	141.0	-	-	-	130.5	-	-	-	138.9	-	-	-	146.2
10	4.20	3H	s	40.4	4.24	3H	s	40.5	4.21	3H	s	40.4	4.20	3H	s	41.0
11	2.89	2H	t (7.4)	29.9	2.91 ₂ /2.91 ₅	2H	2*app t (7.5)	31.8	2.79	2H	t (7.4)	29.6	2.94	2H	t (7.5)	30.2
12	1.81	2H	sext (7.4)	24.8	1.79 ₇ /1.79 ₉	2H	2*app sext (7.5)	25.8	1.70	2H	sext (7.4)	25.0	1.81	2H	sext (7.5)	25.0
13	0.98	3H	t (7.4)	15.9	1.02	3H	t (7.5)	15.9	0.92	3H	t (7.4)	15.8	0.96	3H	t (7.5)	16.0
14	-	-	-	124.7	-	-	-	126.3	-	-	-	128.1	-	-	-	132.1
15	8.24	1H	d (2.4)	133.0	7.82	1H	d (2.4)	132.4	7.81	1H	d (2.4)	132.4	8.00	1H	d (2.5)	133.8
16	-	-	-	130.2	-	-	-	130.0	-	-	-	129.2	-	-	-	128.8
17	7.89	1H	dd (8.9; 2.4)	134.7	7.94	1H	dd (8.9; 2.4)	134.8	7.96	1H	dd (8.9; 2.4)	134.6	7.87	1H	dd (8.8; 2.5)	133.5
18	7.35	1H	d (8.9)	116.3	7.35	1H	d (8.9)	116.0	7.35	1H	d (8.9)	116.0	7.32	1H	d (8.8)	116.4
19	-	-	-	162.8	-	-	-	161.9	-	-	-	162.8	-	-	-	163.9
20	4.33	2H	q (7.0)	68.5	4.23	2H	q (7.0)	68.1	4.21	2H	q (7.0)	68.0	4.21	2H	q (7.0)	67.9
21	1.47	3H	t (7.0)	16.5	1.29	3H	t (7.0)	16.5	1.26	3H	t (7.0)	16.4	1.33	3H	t (7.0)	16.8
24/28 ^{eq} (c)	3.60	2H	dm (11.3)	54.0	3.58	2H	dm (11.2)	54.0/53.9	3.86	2H	dm (13.0)	50.9 ₅ /50.9 ₃	3.87	2H	dm (13.0)	51.0
24/28 ^{ax} (c)	1.94	2H	app t (11.3)	54.0	2.02/1.93	2H	2*app t (11.2)	54.0/53.9	2.46/2.38	2H	2*dd (13.0; 11.4)	50.9 ₅ /50.9 ₃	2.43	2H	dd (13.0; 11.6)	51.0
25/27	2.88	2H	m	52.7	2.90	2H	m	53.0/52.9	3.40	2H	m	54.2/54.3	3.40	2H	m	54.5
29/30	1.01	6H	d (6.4)	20.5	1.04/1.01	6H	2*d (6.5)	20.5	1.26/1.24	6H	2*d (6.8)	17.7	1.24	6H	d (6.6)	17.8
31	-	-	-	-	4.14/3.96	2H	2*qd (15.0; 7.2)	47.8	4.12/3.62	2H	2*qd (14.0; 7.1)	43.5	4.65	2H	q (7.1)	66.4
32	-	-	-	-	1.20	3H	t (7.2)	17.5	1.07	3H	t (7.1)	16.1	1.50	3H	t (7.1)	16.5

	MSildEt ^{N26} (2)				MSildEt ^{N6Et} N ²⁶ (5)				MSildEt ^{O7Et} N ²⁶ (7)

N ^o (a)	δ ¹ H (ppm)		Multiplicity ^(b) (J, Hz)	δ ¹³ C (ppm)	δ ¹ H (ppm)		Multiplicity ^(b) (J, Hz)	δ ¹³ C (ppm)	δ ¹ H (ppm)		Multiplicity ^(b) (J, Hz)	δ ¹³ C (ppm)
3	-	-	-	148.9	-	-	-	148.3	-	-	-	148.3
5	-	-	-	150.9	-	-	-	153.1	-	-	-	158.3
7	-	-	-	157.1	-	-	-	157.2	-	-	-	159.0
8	-	-	-	127.5	-	-	-	127.5	-	-	-	124.1
9	-	-	-	141.1	-	-	-	139.1	-	-	-	146.4
10	4.20	3H	s	40.5	4.23	3H	s	40.4	4.21	3H	s	40.9
11	2.89	2H	t (7.4)	30.0	2.81	2H	t (7.4)	29.6	2.96	2H	t (7.4)	30.2
12	1.80	2H	sext (7.4)	24.9	1.73	2H	sext (7.4)	25.0	1.84	2H	sext (7.4)	24.9
13	0.98	3H	t (7.4)	16.0	0.94	3H	t (7.4)	15.8	0.98	3H	t (7.4)	16.0
14	-	-	-	124.9	-	-	-	129.6	-	-	-	129.1
15	8.22	1H	d (2.4)	133.2	7.78	1H	d (2.4)	132.5	8.01	1H	d (2.5)	133.9
16	-	-	-	130.1	-	-	-	128.0	-	-	-	131.9
17	7.89	1H	dd (8.8; 2.4)	134.9	7.92	1H	dd (8.9; 2.4)	134.4	7.83	1H	dd (8.8; 2.5)	133.3
18	7.35	1H	d (8.8)	116.4	7.33	1H	d (8.9)	115.8	7.31	1H	d (8.8)	116.1
19	-	-	-	163.0	-	-	-	162.4	-	-	-	163.4
20	4.32	2H	q (7.0)	68.6	4.22	2H	q (7.0)	67.9	4.22	2H	q (7.0)	67.7
21	1.47	3H	t (7.0)	16.6	1.28	3H	t (7.0)	16.4	1.35	3H	t (7.0)	16.8
24/28 ^{eq} (c)	3.54	2H	dm (11.3)	54.8	3.52	2H	dm (11.5)	54.8/54.9	3.53	2H	dm (11.2)	54.9
24/28 ^{ax} (c)	2.14	2H	app t (11.3)	54.8	2.17/2.11	2H	2*app t (11.1)	54.8/ 54.9	2.14	2H	app t (11.2)	54.9
25/27	2.74	2H	m	54.9	2.72	2H	m	54.75/54.7	2.73	2H	m	54.8
29/30	1.04	6H	d (6.4)	18.9	1.05/1.02	6H	2*d (6.4)	18.9	1.04	6H	d (6.2)	19.0
31	2.84	2H	q (7.2)	42.5	4.15/3.63	2H	2*qd (14.1; 7.1)	43.4	4.67	2H	q (7.1)	66.2
32	0.86	3H	t (7.2)	8.6	1.09	3H	t (7.1)	16.1	1.51	3H	t (7.1)	16.5
33	-	-	-	-	2.84	2H	q (7.2)	42.4	2.83	2H	q (7.2)	42.5
34	-	-	-	-	0.87	3H	t (7.2)	8.5	0.86	3H	t (7.2)	8.6

	MSildOH (8)				MSildOEt ^{N26} (9) (d)				MSildOEt ^{O7} (10)				MSildOEt ^{O7} Et ^{N26} (11)			
N ^o (a)	δ ¹ H (ppm)	Multiplicity ^(b) (J, Hz)		δ ¹³ C (ppm)	δ ¹ H (ppm)	Multiplicity ^(b) (J, Hz)		δ ¹³ C (ppm)	δ ¹ H (ppm)	Multiplicity ^(b) (J, Hz)		δ ¹³ C (ppm)	δ ¹ H (ppm)	Multiplicity ^(b) (J, Hz)		δ ¹³ C (ppm)
3	-	-	-	147.8	-	-	-	147.7	-	-	-	147.2	-	-	-	147.4
5	-	-	-	155.5	-	-	-	156.7	-	-	-	158.8	-	-	-	158.9
7	-	-	-	158.1	-	-	-	159.1	-	-	-	159.3	-	-	-	159.5
8	-	-	-	127.3	-	-	-	127.6	-	-	-	124.0	-	-	-	124.8
9	-	-	-	141.8	-	-	-	142.2	-	-	-	143.5	-	-	-	143.6
10	4.17	3H	s	40.3	4.18	3H	s	40.4	4.21	3H	s	41.0	4.22	3H	s	41.1
11	2.89	2H	t (7.4)	30.0	2.90	2H	t (7.4)	30.2	2.97	2H	t (7.4)	30.2	2.99	2H	t (7.4)	30.3
12	1.81	2H	sext (7.4)	24.7	1.84	2H	sext (7.4)	24.9	1.85	2H	sext (7.4)	24.6	1.86	2H	sext (7.4)	24.8
13	0.99	3H	t (7.4)	16.0	1.00	3H	t (7.4)	16.2	1.00	3H	t (7.4)	16.0	1.00	3H	t (7.4)	16.1
14	-	-	-	119.3	-	-	-	119.8	-	-	-	122.3	-	-	-	122.5
15	8.59	1H	d (2.7)	132.0	8.50	1H	br	132.3	8.79	1H	d (2.4)	132.2	8.82	1H	d (2.4)	132.4
16	-	-	-	120.3	-	-	-	ND ^(e)	-	-	-	128.7	-	-	-	128.7
17	7.54	1H	dd (8.9; 2.7)	133.7	7.46	1H	dd (8.9; 2.6)	133.7	7.73	1H	dd (8.7; 2.4)	133.9	7.75	1H	dd (8.7; 2.4)	134.2
18	6.83	1H	d (8.9)	125.1	6.75	1H	d (8.9)	125.0	7.18	1H	d (8.7)	121.1	7.20	1H	d (8.7)	121.3
19	-	-	-	176.0	-	-	-	176.3	-	-	-	165.9	-	-	-	166.1
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24/28 ^{eq} (c)	3.81	2H	dm (11.9)	53.7	3.49	2H	dm (11.3)	55.1	3.61	2H	dm (11.2)	54.5	3.57	2H	dm (11.2)	55.0
24/28 ^{ax} (c)	2.32	2H	app t (11.5)	53.7	2.12	2H	app t (11.3)	55.1	1.90	2H	app t (11.0)	54.5	2.15	2H	app t (11.0)	55.0
25/27	3.30	2H	m	53.0	2.73	2H	m	54.9	2.85	2H	m	52.8	2.73	2H	m	54.9
29/30	1.21	6H	d (6.5)	20.1	1.03	6H	d (6.2)	19.1	0.99	6H	d (6.4)	20.8	1.03	6H	d (6.3)	19.1
31	-	-	-	-	2.83	2H	q (7.1)	42.6	4.80	2H	q (7.1)	67.0	4.82	2H	q (7.1)	67.1
32	-	-	-	-	0.86	3H	br t (7.1)	8.7	1.58	3H	t (7.1)	16.3	1.58	3H	t (7.1)	16.3
33	-	-	-	-	-	-	-	-	-	-	-	-	2.82	2H	q (7.2)	42.6
34	-	-	-	-	-	-	-	-	-	-	-	-	0.84	3H	t (7.1)	8.8

	Sild (12)				SildEt ^{N6} (13)				SildOH (14) ^(d)

N ^o (a)	δ ¹ H (ppm)	Multiplicity ^(b) (J, Hz)		δ ¹³ C (ppm)	δ ¹ H (ppm)	Multiplicity ^(b) (J, Hz)		δ ¹³ C (ppm)	δ ¹ H (ppm)	Multiplicity ^(b) (J, Hz)		δ ¹³ C (ppm)
3	-	-	-	149.0	-	-	-	148.3	-	-	-	147.8
5	-	-	-	151.0	-	-	-	153.1	-	-	-	156.1
7	-	-	-	157.2	-	-	-	157.3	-	-	-	158.6
8	-	-	-	127.6	-	-	-	127.6	-	-	-	127.5
9	-	-	-	141.1	-	-	-	139.1	-	-	-	142.0
10	4.19	3H	s	40.6	4.23	3H	s	40.4	4.18	3H	s	40.4
11	2.87	2H	t (7.4)	30.0	2.81	2H	t (7.5)	29.6	2.90	2H	t (7.4)	30.1
12	1.79	2H	sext (7.4)	25.0	1.73	2H	sext (7.5)	25.0	1.83	2H	sext (7.4)	24.9
13	0.97	3H	t (7.4)	16.1	0.94	3H	t (7.5)	15.9	1.01	3H	t (7.4)	16.2
14	-	-	-	125.0	-	-	-	128.0	-	-	-	120.1
15	8.21	1H	d (2.4)	133.2	7.79	1H	d (2.4)	132.4	8.57	1H	br	132.3
16	-	-	-	130.2	-	-	-	129.7	-	-	-	ND ^(e)
17	7.89	1H	dd (8.8; 2.4)	135.0	7.94	1H	dd (8.9; 2.4)	134.5	7.48	1H	dd (8.8; 2.4)	133.7
18	7.34	1H	d (8.9)	116.5	7.34	1H	d (8.9)	115.8	6.77	1H	d (8.8)	125.2
19	-	-	-	163.1	-	-	-	162.4	-	-	-	176.3
20	4.31	2H	q (7.0)	68.7	4.22	2H	q (7.0)	67.9	-	-	-	-
21	1.46	3H	t (7.0)	16.7	1.27	3H	t (7.0)	16.5	-	-	-	-
24/28	3.07	4H	br	48.3	3.05	4H	br	48.1	3.01	2H	br	48.6
25/27	2.56	4H	br	56.2	2.48	4H	br app t (4.7)	56.2	2.48	2H	br app t (4.8)	56.4
29	2.26	3H	s	47.3	2.21	3H	s	47.3	2.20	3H	s	47.5
31	-	-	-	-	4.13/3.64	2H	2*qd (14.1; 7.2)	43.4	-	-	-	-
32	-	-	-	-	1.09	3H	t (7.0)	16.1	-	-	-	-

	DeSild (15) ^(f)				DeSildEt ^{N6} (16) ^(f)

N ^o (a)	δ ¹ H (ppm)	Multiplicity ^(b) (J, Hz)		δ ¹³ C (ppm)	δ ¹ H (ppm)		Multiplicity ^(b) (J, Hz)	δ ¹³ C (ppm)
3	-	-	-	148.7	-	-	-	148.2
5	-	-	-	152.0	-	-	-	153.5
7	-	-	-	157.0	-	-	-	158.5 or 157.4
8	-	-	-	127.2	-	-	-	127.4
9	-	-	-	141.3	-	-	-	139.2
10	4.19	3H	s	40.4	4.22	3H	s	40.3
11	2.89	2H	t (7.4)	29.9	2.79	2H	t (7.5)	29.7
12	1.81	2H	sext (7.4)	24.8	1.73	2H	sext (7.5)	24.9
13	0.99	3H	t (7.4)	15.9	0.94	3H	t (7.5)	15.9
14	-	-	-	123.2	-	-	-	124.8
15	8.10	1H	dd (7.8; 1.7)	133.3	7.37	1H	dd (7.4; 1.8)	132.6
16	7.15	1H	td (7.5; 1.0)	124.0	7.12	1H	td (7.5; 0.9)	123.4
17	7.55 ₂	1H	ddd (8.5; 7.4; 1.8)	135.5	7.54 ₉	1H	os	134.2
18	7.19	1H	d (8.5)	115.9	7.16	1H	os	115.1
19	-	-	-	159.4	-	-	-	158.5 or 157.4
20	4.27	2H	q (7.0)	67.8	4.11	2H	q (7.0)	66.8
21	1.48	3H	t (7.0)	16.7	1.23	3H	t (7.0)	16.7
31	-	-	-	-	4.15/3.65	2H	2*qd (14.0; 7.0)	43.1
32	-	-	-	-	1.06	3H	t (7.0)	15.9

^a Position number indicates either hydrogen or carbon.

^b Multiplicity: s, singlet; d, doublet; dd, doublet of doublet; ddd, doublet of doublet of doublet; t, triplet; td, triplet of doublet; q, quartet; qd: quartet of doublet; sext: sextet; m: multiplet; dm: doublet of multiplet; app: apparent; br: broad; os overlapped signal.

^c ax: axial; eq: equatorial.

^d Due to the low solubility of the compound, δ were measured in CD₃CN:D₂O (80:20) after basification of the medium by 5 μ L of 0.5 M NaOD added in the NMR tube.

^e ND: not detected.

^f Compounds **15** and **16** were not separated.

Isolation and identification of ten new sildenafil derivatives in an alleged herbal supplement for sexual enhancement

Supplementary information

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(B)

Pip A: 2,6-dimethylpiperazine

Pip B: N1-ethyl-2,6-dimethylpiperazine

Pip C: N-methylpiperazine

(c)

m/z
298 **326**

m/z without ethyl group on the pyrazolopyrimidine moiety

459

Pip A: 2,6-dimethylpiperazine

487

Pip B: N1-ethyl-2,6-dimethylpiperazine

445

Pip C: N-methylpiperazine

m/z with ethyl group

487

515

Figure S2. Parts of 2D NOESY spectra showing the spatial correlations between CH₂ 11, 12 and H31 for compound **3** (MSildEt^{N4}, N4-ethyl-methisosildenafil) and between H15 and CH₂ 31 for compound **4** (MSildEt^{N6}, N6-ethyl-methisosildenafil).

Figure S3. Representation of a privileged conformation calculated for compound **3** (MSildEt^{N4}, N4-ethylmethisosildenafil) and interatomic distances between protons of interest.

	H31a-H11a	3.77 Å	H31a-H12a	3.88 Å	
H31a-H15	4.97 Å	H31a-H11b	2.27 Å	H31a-H12b	2.38 Å
H31b-H15	3.88 Å	H31b-H11a	5.36 Å	H31b-H12a	5.46 Å
	H31b-H11b	3.82 Å	H31b-H12b	3.87 Å	

Figure S4. Part (6.7-8.9 ppm) of the ^1H NMR spectrum with ^{13}C GARP broadband decoupling of the dietary supplement recorded in $\text{CD}_3\text{CN}/\text{D}_2\text{O}$ (80:20, v/v) with attribution of the signals of the identified compounds.

(A) **(B)** **(C)**