

## Optimization of Extraction Methods for Determination of Phosphodiesterase-5 (PDE5) Inhibitors in Premix Coffee

(Pengoptimuman Kaedah Pengekstrakan bagi Penentuan Perencat Fosfodiesterase-5 (PDE5) dalam Kopi Pracampuran)

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### ABSTRACT

*An efficient analytical technique capable of analyzing three most common phosphodiesterase-5 (PDE5) inhibitors (varidenafil, sildenafil and tadalafil) simultaneously in premix coffee was developed. Sample extractions using either acetonitrile or methanol with two different extraction techniques (with and without evaporation steps) were evaluated. Identification and quantitation was conducted by high performance liquid chromatography with photo-diode-array (HPLC-DAD) at different wavelengths; 230 nm, 245 nm and 290 nm; and by time of flight mass spectrometry (LC-MS-TOF). Extraction with acetonitrile (without evaporation with nitrogen) showed recovery ranging from 105% to 113% ( $\pm <10\%$ ) for HPLC-DAD at 245 nm and 93% to 102% ( $\pm <2.5\%$ ) for LC-MS-TOF. Chromatogram separation was best achieved with mobile phase consisted of water (0.1% formic acid) and acetonitrile (0.1% formic acid) with gradient elution within 20 min. Thus, the results indicated that extraction using acetonitrile without evaporation step was the most efficient technique for determination of PDE5 inhibitors in premix coffee.*

*Keywords: HPLC-DAD; LC-MS-TOF; optimization; PDE5 inhibitors; premix coffee*

### ABSTRAK

*Satu kaedah pengekstrakan yang ringkas dan cekap telah berjaya dibangunkan untuk mengesan kehadiran perencat fosfodiesterase-5 (PDE5) iaitu vardenafil, sildenafil dan tadalafil dalam produk kopi pracampuran. Pengekstrakan sampel menggunakan asetonitril atau metanol dengan dua kaedah pengekstrakan (melalui dan tanpa melalui penyejatan bernitrogen) dinilai. Penentuan dan kuantifikasi sebatian adalah menggunakan kromatografi cecair prestasi tinggi (HPLC) dengan pengesan susunan diod (DAD) pada panjang gelombang 230 nm, 245 nm dan 290 nm serta kromatografi cecair spektrometri jisim masa penerbangan (LC-MS-TOF). Pengekstrakan sampel menggunakan asetonitril (tanpa melalui penyejatan bernitrogen) menunjukkan peratusan perolehan semula antara 105% hingga 113% ( $\pm <10\%$ ) dengan HPLC-DAD pada panjang gelombang 245 nm dan 93% hingga 102% ( $\pm <2.5\%$ ) dengan LC-MS-TOF. Pemisahan kromatogram paling baik diperolehi dengan kombinasi fasa bergerak air (0.1% asid formik) dan asetonitril (0.1% asid formik) dengan kecerunan elusi dalam tempoh 20 min. Ini membuktikan kaedah pengekstrakan sampel menggunakan asetonitril tanpa langkah penyejatan bernitrogen merupakan teknik yang paling cekap dalam menentukan perencat PDE5 dalam produk kopi pracampuran.*

*Kata kunci: HPLC-DAD; kopi pracampuran; LC-MS-TOF; pengoptimuman; perencat PDE5*

### INTRODUCTION

Synthetic phosphodiesterase type 5 enzyme (PDE5) inhibitors, namely sildenafil citrate (Viagra®, manufactured by Pfizer), vardenafil hydrochloride (Levitra®, manufactured by Bayer) and tadalafil (Cialis®, manufactured by Lilly) are widely used for the treatment of erectile dysfunction (Reepmeyer & d'Avignon 2009). It was reported that the use of PDE5 inhibitors have some side effects especially in patients taking nitrates and therefore it has to be used under strict medical supervision (Eardley & Sethia 2003; Stehlik & Movsesian 2009).

Herbal formulation, which has been generally believed safe to be consumed, is thus an option to remedy the problem compared to synthetic drugs (Savaliya et al. 2010).

However recently, the adulteration of PDE5 inhibitors found in herbal products claimed to be natural products for the treatment of erectile dysfunction have been reported in many countries. Gratz et al. (2004) reported in United States, almost 50% of herbal products analyzed were adulterated with either sildenafil, tadalafil or its analogues. Fleshner et al. (2005) found that 2 out of 7 herbal products marketed in Canada were contaminated with sildenafil and tadalafil. In China, 28 from 80 herbal samples for sexual enhancement being analyzed were also found to be adulterated with sildenafil (Liang et al. 2005).

Various extraction methods have been developed to determine the presence of PDE5 inhibitors and its analogues in pharmaceutical and herbal products (Mikami

et al. 2002; Venhuis et al. 2008; Zou et al. 2008). Various extraction techniques with different types of solvents were reported. Bogusz et al. (2006) stated that herbal capsules was extracted in methanol, centrifuged and filtered before instrumentation. Reepmeyer and d'Avignon (2009) reported that the sample was extracted with methanol, then being evaporated and dissolved in isopropanol. On the other hand, Zou et al. (2006) mentioned that sample was extracted with acetonitrile, filtered and then injected into instrument. Other solvents were also used such as ethanol (Fleshner et al. 2005; Mikami et al. 2002), combination of dichloromethane, isopropanol and methanol (Bogusz et al. 2006) and chloroform and methanol (Zou et al. 2008).

High performance liquid chromatography (HPLC) was found to be the most common instrument used in determining PDE5 inhibitors and its analogue. Reports were published on the use of HPLC with detector of either UV (Cheng & Chou 2005; Zhu et al. 2005), photo-diode-array (Choi et al. 2008; Reepmeyer & Woodruff 2007), fluorescence (Cheng et al. 2007) or mass spectrometry (Liang et al. 2005; Venhuis et al. 2008, Zou et al. 2006).

In Malaysia, monitoring on adulterations of PDE5 inhibitors has been conducted on traditional products (National Pharmaceutical Control Bureau Report 2009). However, there was a great concern of whether the adulteration occurred in premix coffee were added with similar type of herbs. Currently no methodologies are available to detect these adulterants in food products, specifically in premix coffee. Thus the objective of this study was to optimize a simultaneous detection method of vardenafil, sildenafil and tadalafil by HPLC-DAD and LC-MS-TOF in premix coffee sample.

## MATERIALS AND METHODS

### MATERIALS

Sildenafil citrate and tadalafil used in this study were obtained from Toronto Research Chemical, Canada and vardenafil hydrochloride were from PharmaChem, Canada. Methanol, acetonitrile (analytical grade) and formic acid (reagent grade) were purchased from Merck.

### SAMPLES

Blank premix coffee samples are prepared in the laboratory. Decaffeinated coffee (Nescafe Gold), non-dairy creamer (Tesco) and coarse sugar (Central Sugars Refinery) were purchased from local supermarket. The blank samples are prepared by mixing decaffeinated coffee (50 g), coarse sugar (100 g) and non-dairy creamer (150 g) with a ratio of 1:2:3 and mechanically powdered (1093 Cyclotec Sample Miller, Sweden) to get fine powder form.

### INSTRUMENTS

The chromatographic analysis was performed using HPLC (Shimadzu, Kyoto, Japan) system equipped with on-line

degasser (DGU-14A), low pressure pump (LC-10AD), photo-diode-array (DAD) detector (SPD-M10A), auto injector (SIL-10AD) and column oven (CTO-10A). Data were analysed using CLASS-VP software of version 5.33. The chromatographic separation of compounds was achieved with a reversed phase column, Zorbax Extend C18 (3.5  $\mu\text{m}$ , 150 mm  $\times$  4.6 mm i.d) from Agilent, operating at 40°C and a flow rate of 1 mL/min was applied. Injection sample volume was 20  $\mu\text{L}$ .

The mass spectrometry system used was the Agilent 6210 time-of flight LC-MS (Agilent, Littlefall, USA) with LC part consist of binary pump, auto sampler, degasser and column compartment; all from 1200 series (Agilent Waldbronn, Germany). Mass Hunter Workstation software (version B.01.04.) was used for data analysis. The data were recorded within range from 100 and 1000 m/z. Reversed phase column was a Zorbax Extend C18 (50 mm  $\times$  3 mm with 1.8  $\mu\text{m}$  particle size) with mobile phase flow rate at 0.2 mL/min and sample injection volume of 5  $\mu\text{L}$ .

### METHOD PROCEDURES

Recovery experiments were performed to evaluate the reliability and suitability of the optimized conditions of PDE5 inhibitors in premix coffee. In this study, blank premix coffee powder was spiked with 10  $\mu\text{g/g}$  standard concentration. Two different types of extraction solvents which were acetonitrile (A) (Gratz et al. 2004; Zou et al. 2006) and methanol (M) (Choi et al. 2008; Liang et al. 2006; Zou et al. 2008) with two different extraction steps; without nitrogen evaporation (Gratz et al. 2004) and with nitrogen evaporation step as used by Bogusz et al. (2006) were evaluated.

#### EXTRACTION WITHOUT EVAPORATION STEP

A 1 g sample was weighed into 50 mL centrifuge tube, added with 10 mL solvent and being shake by overhead shaker (Reax 2, Heidolph, Germany), sonicated (S 60 H Elmasonic, Elma, Germany) and centrifuged (Universal 32R, Hettich Zentrifugen, Germany) at 4000 rpm. The supernatant was then filtered with 0.2  $\mu\text{m}$  PVDF micro filter and injected into instruments. Result collected from this method were labelled as A1 (using acetonitrile as extraction solvent) and M1 (using methanol as extraction solvent).

#### EXTRACTION WITH EVAPORATION STEP

The first part of the extraction were the same as above mentioned, however following the centrifugation step, the sample supernatant collected was further evaporated using nitrogen evaporator (N-Evap 112, Organomation, USA) until complete dryness, reconstituted with 1 mL acetonitrile: water (1:1), filtered and ready for instrumentation. Result collected from this method were labelled as A2 (using acetonitrile as extraction solvent) and M2 (using methanol as extraction solvent).

#### OPTIMIZATION OF HPLC

Separation of vardenafil, sildenafil and tadalafil were attempted using two different combinations of mobile phase: The combination of water and acetonitrile (Reepmeyer & Woodruff 2007) with modification on gradient elution programme to obtain the best separation and the combination of water and methanol (Reepmeyer & d'Avignon 2009) with modification on gradient elution programme to obtain the best separation.

The selected combination of mobile phase was further optimized by two different matrix modifier to find the most suitable matrix modifier in this analysis: 10 mM ammonium formate as reported by Bogusz et al. (2006) and 0.1% formic acid (Mikami et al. 2002).

Chromatogram data were collected from 190 nm to 500 nm wavelength, within 20 min of run time. The UV signal were monitored at 230 nm (Reepmeyer & d'Avignon 2009), 245 nm and 290 nm (Zou et al. 2006).

#### OPTIMIZATION OF LC-MS-TOF

The developed HPLC method was then transferred to LC-MS-TOF analysis but the gradient programme was optimized to get best separation. The mass spectrometer was operated in the positive ion mode as recommended by Wang et al. (2009). Nitrogen gas was utilized as the nebulizer gas and electrospray ionization (ESI)-low concentration tuning mix solution (Agilent G1969-85000) used as the calibrations solution. All the masses were corrected using internal reference ions of  $m/z$  322.0481, 622.0289 and 922.0098 with mass error less than 5 ppm.

#### STATISTICAL ANALYSIS

The results were expressed as mean value  $\pm$  standard deviation of three replicates. Analysis of sample was performed using SPSS software version 11.5 2002 to determine the variation of recovery among four test methods and individual analyte. The level of significance was  $p < 0.05$ .

### RESULTS AND DISCUSSION

#### SELECTION OF MOBILE PHASE FOR HPLC ANALYSIS

The combination of water and methanol as the mobile phase are able to separate well tadalafil from vardenafil and sildenafil with high intensities. However, the background noise is noticeably high and affect the identification of the analytes of interest. The same finding was reported by Gratz et al. (2004). The peak of vardenafil and sildenafil however are not separated well with the use of methanol as both analytes are very similar in the molecular structure and its polarity. Zhu et al. (2005) reported that the peak of vardenafil and sildenafil are not well separated when methanol is used as mobile phase.

The use of acetonitrile and water however provide better separation with high intensities for all three

compounds. All three peaks of vardenafil, sildenafil and tadalafil were distinguishably separated. The peak of caffeine is also separated from the three interested compounds. At the same time, the background noise is not too significant as to interfere with the peak of interest.

The presence of several N atoms in the molecular structure of target compounds would cause serious peak tailing if no modifier was added into mobile phase in HPLC analysis (Wang et al. 2009). Ammonium formate was used as matrix modifier by Bogusz et al. (2006) to separate sildenafil, tadalafil and other drugs in herbal products by LC-MS/MS. It was found in this study that the use of ammonium formate was not suitable to separate both sildenafil and vardenafil by HPLC. Peaks of vardenafil and sildenafil found to be overlapping with each other and these two compounds failed to be separated either by isocratic or gradient elution. The use of ammonium formate was presumably suitable to analyze both sildenafil and vardenafil simultaneously if using mass spectrometry because the chromatogram of each target compound can be extracted individually, but not with DAD detector.

The separation problem was resolved by adding formic acid in mobile phase as recommended by Mikami et al. (2002). It was found that the use of 0.1% formic acid was able to separate these two peaks of sildenafil and vardenafil. It was reported that mobile phase in acidic condition help to improve the sensitivity of sildenafil in liquid chromatography (Mikami et al. 2002). As a conclusion, the use of acetonitrile and water with 0.1% formic acid as matrix modifier added to both solution was found to be the most suitable mobile phase in this study.

#### OPTIMIZATION OF GRADIENT ELUTION

A linear solvent gradient using 0.1% formic acid in water and 0.1% formic acid in acetonitrile with flow rate of 1 ml/min was the most suitable combinations in terms of analysis time, resolution and sensitivity. Best separation obtained when composition of 0.1% formic acid in acetonitrile was linearly ramped from 3% to 80% in 10 min and being maintained within 5 min before dropped instantly to 3% for another 5 min. Prominent peaks of vardenafil, sildenafil and tadalafil were resolved at 8.459 min, 9.109 min and 11.157 min, respectively, as shown in Figure 1(a). The compounds were identified by comparing the retention time with the individual compounds retention time (Abdel-Hamid 2006).

#### WAVELENGTH SELECTION

The wavelengths of 290 nm and 230 nm were commonly selected to quantitate PDE5 inhibitors in pharmaceutical and herbal products (Aboul-Enein & Ali 2005; Reepmeyer & d'Avignon 2009). It was found that at 290 nm the peak intensities of all analytes were highest as compared with 230 nm and 245 nm. However in premix coffee sample, interferences signal was also higher and made quantification more difficult as it is very close to the

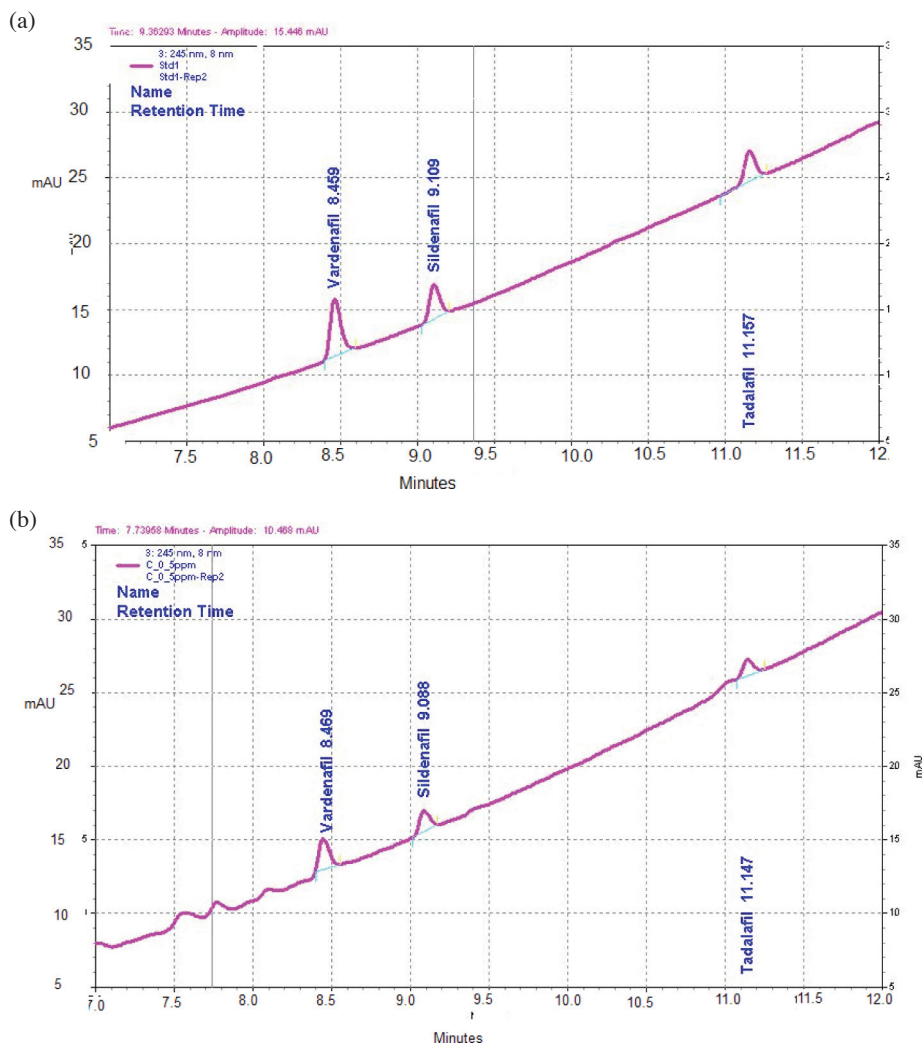


FIGURE 1. The HPLC chromatograms (245 nm) of vardenafil, sildenafil and tadalafil in (a) standard solutions and (b) spiked at 0.5 mg/kg

compound of interest. The intensities of background matrix interference were less at 230 nm and yet peak intensities of analytes of interest were very small. Thus, the identification and quantitation of vardenafil, sildenafil and tadalafil in premix coffee was selected at 245 nm as it provided prominent peak intensities with least matrix interferences and more stable baseline.

#### OPTIMISATION OF LC-MS-TOF

Based on previous finding of HPLC, the mobile phase selected was used with some modification on gradient programme to best separate the compound in LC-MS-TOF. The results showed that an initial condition of 3% of 0.1% formic acid in acetonitrile, which was gradually increased to 65% in 3 min, kept for 12 min before returned to the initial condition and sustained for 5 min before the next injection, found to be the best conditions to separate all compounds. The mass spectrometer was operated in the positive ion mode as the responses of the studied targets under the positive mode were much better than negative mode (Wang et al. 2009). Ion chromatograms of vardenafil,

sildenafil and tadalafil were extracted at  $m/z$  475.2118, 489.2286 and 390.1461, respectively (Figure 2).

MS parameters were optimized by adjusting four major ESI parameters: the capillary voltage, the nebulizer gas pressure, the drying gas flow rate and the fragmentor voltage (Gratz et al. 2004). The same finding was observed as stated by Gratz et al. (2004) that the variations of intensities were not significant when the capillary voltage, nebulizer gas pressure and drying gas flow rate were varied. The conditions of capillary voltage, nebulizer gas pressure and drying gas flow rate were adapted from the recommended setting of the instruments, that are 4000 V for capillary voltage, 2.75 bar for the nebulizer gas pressure and 10 L/min drying gas flow rate. The other parameters for LC-MS-TOF were adjusted and optimized to enhance the intensities of the peak of interest and the optimized conditions were 350°C for gas temperature, octapole RF 250 V and skimmer 60 V.

Several fragmentor voltages were applied and the resulting mass spectra were observed. In this study, the fragmentor voltage of less than 100 V was not enough to

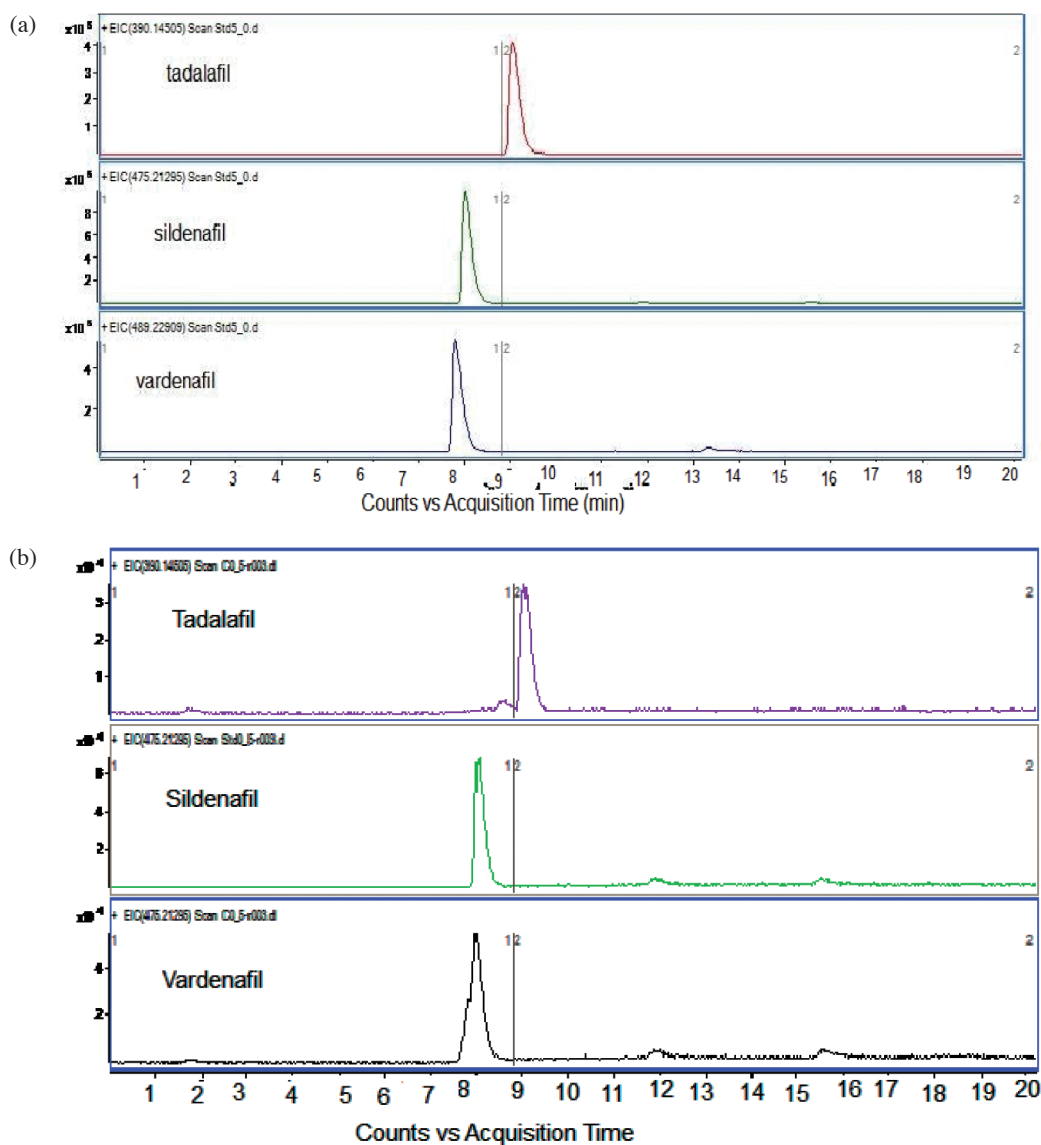


FIGURE 2. The extracted ion chromatograms from LC-MS-TOF of tadalafil, sildenafil and vardenafil (a) standard solutions and (b) spiked sample at 0.5 mg/kg

fragment all compounds. At fragmentor voltage of 120 V, sildenafil and vardenafil were fragmented with dominant peak of the molecular ion  $[M+H]^+$  which were 475.2118 and 489.2286, respectively. However, no fragmentation was observed for tadalafil. Fragmentation of tadalafil was observed when the fragmentor voltage was set at 180 V, with the molecular ion  $[M+H]^+$  of 390.1461 was still dominant, however it exhibited extensive fragmentation for sildenafil and vardenafil and the molecular ion were no longer observed. Thus, a time segment with 120 V was chosen for vardenafil and sildenafil and 180 V for tadalafil. These fragmentor voltages were selected for this analysis because it allowed the formation of structurally useful fragment ions while maintaining sufficient  $[M+H]^+$  response for all of the three compounds studied (Gratz et al. 2004). Figure 3 illustrates the spectra of individual analyte of tadalafil, sildenafil and vardenafil as (a), (b) and (c), respectively.

#### OPTIMIZATION OF SAMPLE EXTRACTION

Two different extraction techniques; without the evaporation step and with evaporation step were applied in order to choose the most efficient technique to analyze premix coffee sample. On both methods, two type of extraction solvent were tested, acetonitrile (A) and methanol (M).

Method 1 was previously developed and applied by Gratz et al. (2004) to the analysis of herbal products. However, premix coffee contains more matrix interferences such as fat, caffeine, sugar and we presumed much lower concentration of adulterant added (if any) as compared to pharmaceutical and herbal products. Thus, the methodology was optimised in order to improve the efficiency for our samples. Modification aimed to obtain more concentrated extract, thus the amount of sample weighed were increased to one gram and the final extract was not diluted as recommended.

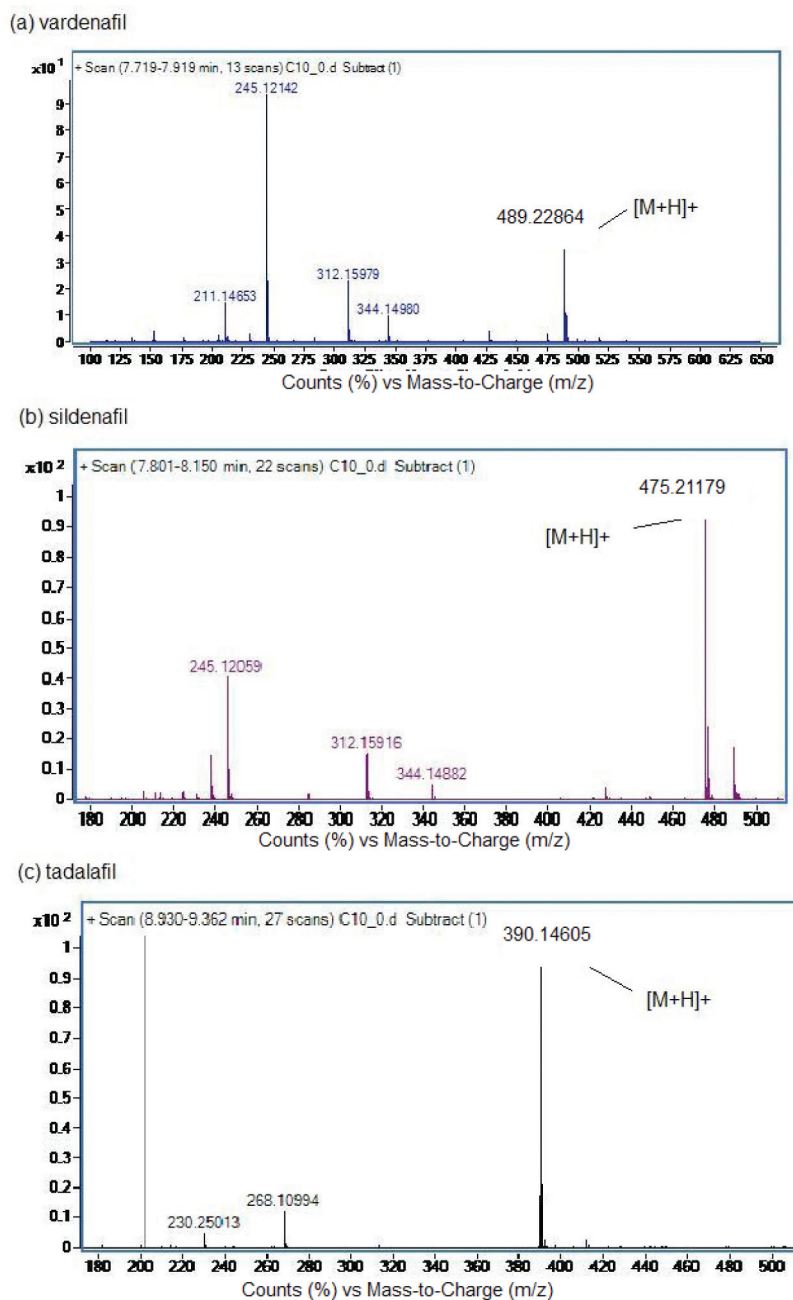


FIGURE 3. Spectra of (a) vardenafil (b) sildenafil and (c) tadalafil by LC-MS-TOF

Method 2 was established and reported by Bogusz et al. (2006) on herbal remedies in tablet and liquid form. Sample containing sugars (herbal honey) were extracted with dichloromethane: isopropanol (9:1), centrifuged and the supernatant was evaporated and reconstituted with methanol prior to instrumentation. The only modification made on this method was the replacement of dichloromethane: isopropanol (9:1) with either acetonitrile or methanol as extraction solvent, since most studies reported the efficiency of these solvents in the analysis of PDE5 inhibitors (Reepmeyer & d'Avignon 2009; Zou et al. 2006).

The mean recoveries and standard deviation for all four methods attempted is illustrated in Tables 1 and 2.

All analytes by HPLC-DAD displayed recovery of more than 80% and 30% to 102% from LC-MS-TOF. The standard deviation was less than 10% for all samples extracted and detected by LC-MS-TOF, but only sample extracted with acetonitrile (Method A1 and A2) demonstrated standard deviation of less than 10% by HPLC-DAD.

Methanol was found not suitable to extract PDE5 inhibitors in premix coffee, as it generates very high interferences with DAD detection. This findings support the statement made by Gratz et al. (2004) that acetonitrile proved a good recovery results as compared with the methanol which generated higher background noise. The report also stated that acetonitrile was necessary solvent for tadalafil due to its poor solubility in methanol.

TABLE 1. Extraction method performance based on recovery and standard deviation of spiked samples ( $n=3$ ) for HPLC-DAD

Analyte	Method A1	Method M1	Method A2	Method M2
Tadalafil	112.9 ± 8.0 <sup>c</sup>	140.4 ± 15.6 <sup>b</sup>	80.7 ± 6.6 <sup>d</sup>	222.9 ± 16.6 <sup>a</sup>
Sildenafil	105.5 ± 9.2 <sup>c</sup>	744.0 ± 55.0 <sup>b</sup>	82.3 ± 3.5 <sup>c</sup>	1389.0 ± 71.7 <sup>a</sup>
Vardenafil	110.9 ± 9.0 <sup>b</sup>	419.0 ± 26.8 <sup>a</sup>	101.3 ± 3.0 <sup>b</sup>	NA*

\* result were not calculated due to a very high interferences

Different alphabets within the same row indicate significant different ( $p<0.05$ )

A – extraction with acetonitrile

M – extraction with methanol

1 – extraction without evaporation step

2 – extraction with evaporation step

TABLE 2. Extraction method performance based on recovery and standard deviation of spiked samples ( $n=3$ ) for LC-TOF-MS

Analyte	Method A1	Method M1	Method A2	Method M2
Tadalafil	96.9 ± 0.8 <sup>a</sup>	87.6 ± 2.7 <sup>b</sup>	83.5 ± 3.3 <sup>b</sup>	30.4 ± 1.7 <sup>c</sup>
Sildenafil	93.3 ± 1.2 <sup>a</sup>	80.2 ± 1.8 <sup>b</sup>	77.2 ± 3.2 <sup>b</sup>	34.2 ± 1.6 <sup>c</sup>
Vardenafil	101.7 ± 2.5 <sup>a</sup>	90.4 ± 2.0 <sup>b</sup>	73.1 ± 1.7 <sup>c</sup>	32.8 ± 1.6 <sup>d</sup>

Different alphabets within the same row indicate significant different ( $p<0.05$ )

A – extraction with acetonitrile

M – extraction with methanol

1 – extraction without evaporation step

2 – extraction with evaporation step

LC-MS-TOF result's showed that all compounds were detected and separated well from interferences, on both extractions either by methanol or acetonitrile except for method M2 which showed very poor recovery for all analytes. This may be due to the mass accuracy featured by LC-MS-TOF, which enabled the system to extract individual compound with mass error of less than 5 ppm.

#### EVALUATION OF METHOD PERFORMANCE

Statistical analysis showed that all four extraction methods were significantly different ( $p<0.05$ ) for both instruments, HPLC-DAD and LC-MS-TOF. Further statistical analysis found that tadalafil was significantly different ( $p<0.05$ ) for all four methods detected with HPLC-DAD as can be seen in Table 1. However, sildenafil and vardenafil were not differently significant ( $p>0.05$ ) by method A1 and A2. Result of LC-MS-TOF showed that tadalafil and sildenafil were not significantly different ( $p>0.05$ ) for method M1 and A2, yet differ significantly ( $p<0.05$ ) for method A1 and M2 as indicated in Table 2. Vardenafil found to be significantly different ( $p<0.05$ ) for all four methods.

According to the guideline on the acceptance of method performance by European Union (2002/657/EC), the accuracy must be within 80% and 110% for sample spiked at concentration of 10 µg/g. Therefore, methods A1 and M1 fulfilled the criteria for acceptance of performance using LC-MS-TOF whereas method A1 and A2 by HPLC-DAD.

Methods A1 and A2 were finally selected for additional statistic analysis due to quantitation to be conducted by HPLC-DAD. Based on statistical test between obtained result and target value (100%), it was proven that there

was no significant difference ( $p>0.05$ ) for all analytes extracted by method A1 by HPLC-DAD. However, method A2 showed there were no significant different ( $p>0.05$ ) only for vardenafil, whereas sildenafil and tadalafil were significantly different ( $p<0.05$ ).

Based on the presented results, method A1 was finally selected for future studies to determine the presence of tadalafil, sildenafil and vardenafil simultaneously in premix coffee. Furthermore, the extraction technique was faster as compared with method A2, which took 3 h longer. Figure 1(b) illustrates a HPLC chromatogram of spiked sample at concentration of 0.5 mg/kg which was extracted using method A1 and Figure 2(b) for LC-MS-TOF extracted ion chromatogram.

#### CONCLUSION

The result of this study showed that an extraction with acetonitrile without the evaporation step was the best extraction technique to detect sildenafil, tadalafil and vardenafil simultaneously in premix coffee. All three analytes were best separated and quantified either by HPLC-DAD (245 nm) or LC-MS-TOF using combination of 0.1% formic acid in water and 0.1% formic acid in acetonitrile as mobile phase. However, LC-MS-TOF provided better recovery and lower standard deviation. This rapid and economic methodology can be applied to premix coffee and similar matrices.

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