SUPPLEMENTARY MATERIAL

Optimisation of a Naviglio-assisted extraction followed by determination of piperine content in *Piper longum* extracts

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Studies were made to increase the yield of piperine extraction using Naviglio Extractor[®] (SLDE, Solid-Liquid Dynamic Extrction) from fruit of *Piper longum*. The effects of ratio w/v were investigated and optimized for the best method. The maximum yield of piperine (317.7 mg/g) from *Piper longum* fruits was obtained in SLDE 1:50 ethanol extract. Extraction yields of piperine obtained from Soxhlet (SE), decotion (ISO) and conventional maceration (CM) extraction methods were found to be 233.7 mg/g, 231.8 mg/g and 143.6 mg/g, respectively. The results of the present study indicated that Naviglio Extractor[®] is an effective technique for the extraction of piperine from long pepper.

Keywords: *Piper longum*, extraction, Naviglio Extractor[®], piperine, UV-Vis determination

1. Experimental

1.1. General experimental procedures

Piperine standard was purchased from Sigma-Aldrich (Milan, Italy). All solvents for extraction and UV analyses were of analytical grade purity and were purchased from VWR (Milan, Italy). Hexane (H) and *i*-propanol (IPA) (HPLC grade) by VWR were used for the mobile phase and for sample preparation in HPLC analyses. Deionized water was purified by a Milli-Q water purification system from Merck-Millipore.

A dynamic solid–liquid extraction was carried out using a Naviglio Extractor[®] mod. 1000cc (Atlas Filtri Engineering Srl, Padua, Italy).

Quantitative analyses of piperine were determined using an UV/Vis T70+ spectrophotometer (PG Instruments Ltd.) operating with 1nm spectral bandwidth, ± 0.3 nm wavelength accuracy and splitbeam optical system. Quartz cells with 1 cm optical path length were used.

The HPLC analyses were carried out on a HPLC HP 1100 instrument equipped with a quaternary pump and coupled to a UV-Vis detector (254 nm). For compound separation the HPLC instrument was equipped with a semipreparative Luna 5 μ Silica (2) 100A column (Phenomenex) (100 x 4.6 mm) kept at 25 °C, a loop of 200 μ L, for the elution isocratic H-IPA 96:4 and 4mL/min flow rate were used. For the analysis of the extracts an Agilent LiChrospher Si60 (250 x 4 mm) column kept at 25 °C and a loop of 20 μ L were used. The elution was carried out in an isocratic system (H-IPA 96:4) and flow rate 1 mL/min.

The isolated compounds were analysed by ¹H and ¹³C NMR (acquired by using a JMODXH pulse sequence). Spectra were recorded in CDCl₃ at 400 and 100 MHz on a Bruker Avance-DRX 400. Chemical shifts (δ) were determined in ppm relative to residual solvent signals (CHCl₃, 7.26 ppm for ¹H NMR, CDCl₃, 77.0 ppm for ¹³C NMR).

TLC (Thin Layer Chromatography) was performed on Merck Silica gel 60 F₂₅₄ plates and spots were visualized under UV light and after staining with p-anisaldehyde-H₂SO₄-EtOH (1:1:98) followed by heating at 110 °C.

1.2. Plant material.

Dried fruits of *P. longum* L. purchased from Demar srl (Cesena, FC, Italy) collected in 2014 in Indonesia (lot 191/05/14/243) were generously provided by Aboca S.p.A. (Sansepolcro, Italy).

1.3. Extraction methods

1.3.1. Decotion (ISO). According ISO rules (ISO 1982), 0.5 g of ground fruits of *P. longum* were extracted with 50 mL of 96% EtOH and refluxed for 3 h avoiding light. The extraction mixture was allowed to cool and filtered using Whatman No. 1 filter paper. The resulting solution was evaporated under reduced pressure.

1.3.2. Maceration (CM). Ground piper fruits (0.5 g) were extracted with 50 mL of 96% EtOH, and then the solution was kept at room temperature avoiding light. The extraction time was set at 3 h. After extraction, the suspension was filtered using Whatman No. 1 filter paper. The resulting solution was evaporated under reduced pressure.

1.3.3. Soxhlet extraction (SE). Ground piper fruits (1.5 g) were extracted for a total time of 3 h using 150 mL of solvent at the reflux temperature avoiding light. At the end of the process, the extract was cooled at room temperature and evaporated under reduced pressure.

1.3.4. Naviglio (SLDE). Naviglio is an apparatus that allows a fast solid–liquid extraction by means of a programmable pressurization cycle of the liquid in contact with the solid between atmospheric pressure and 8–9 bar (Naviglio 2003). The Naviglio extraction is based on a suction effect generated by a compression of the solvent on solids at room temperature and a settled pressure, followed by immediate decompression at atmospheric pressure (Naviglio *et al.*, 2014). The conditions used for the extractions were as follows: operating pressure 8 bar, total cycles 20, total duration of the extraction process 3 h, 12 shots in the swing phase, duration of the static phase 2 min, and duration of the dynamic phase 2 min. Ground piper fruits (6.0 g) were placed inside the extraction chamber in a food-grade polyethylene bag with a porosity of 50 µm, and 600 mL of solvent were added. At the end of the process (3 h), the extract was recovered and evaporated under reduced pressure (SLDE 1:100 extract). In order to study the influence of drug: solvent ratio on the yield of piperine extraction, we performed other two extractions using 12 g and 60 g of *Piper longum* in a 1:50 (SLDE 1:50) and 1:10 (SLDE 1:10) w/v ratio with solvent. The extractions were performed in the same manner.

The reported yields are the mean of the three different extractions with their SD.

1.4. Isolation and characterization of principal metabolites.

For the isolation of the compounds, 200 µL of a 15 mg/mL solution of the SLDE 1:100 extract in a 8:2 H-IPA solution were injected and main compounds were collected and identified. Compounds were identified by comparison of their spectral data with that in the literature (Li *et al.*, 2013).

1.5. Qualitative analyses of the extracts.

Stock solutions of the extracts at the concentration of 1 mg/mL in H-IPA 8:2 were sonicated and filtered through 0.45 µm nylon syringe membranes prior to use. The resulting clear solutions (1 mL) were diluted to 5 mL by H-IPA 8:2 and 20 µL were injected.

1.6. Quantitative determination of piperine.

Stock solutions of the extracts at the concentration of 1 mg/mL in absolute EtOH were sonicated and filtered through 0.45 µm nylon syringe membranes prior to use. The resulting clear solutions (0.2 mL) were diluted to 10 mL. All the UV spectra were recorded at 343 nm.

1.7. Validation of the analytical method.

The method was validated for linearity, limits of detection (LOD), limits of quantitation (LOQ), precision and recovery, according to the International Conference on Harmonisation (ICH) Guidelines (ICH, 2005). All statistical analyses conducted in this study were performed using an Excel spreadsheet (Microsoft Co., USA) software.

1.7.1. Calibration method.

The amount of piperine in the prepared extracts was determined according to the linear calibration curves of the target standard. Five point calibration curve for piperine was constructed for the quantification of the compound. For the calibration standard, stock solution (1 mg/mL in EtOH) was diluted with absolute EtOH to produce standard working solutions at concentrations of 2.5, 3.75, 5.0, 7.50 and 10 µg/mL of piperine. The absorbance values were the average of three replicate analyses.

1.7.2. Precision. To evaluate the repeatability of the method, one sample of a 6 µg/mL solution of piperine was analysed for two non consecutive days. Precision levels were calculated by the relative standard deviation percentage (RSD%) from the analytical curves.

1.7.3. Accuracy. The accuracy of the UV method method was established in the analytical range for piperine by analyzing standard solutions at three concentrations of piperine (4, 6, 8 µg/mL). The accuracy (recovery %) was determined using equation 3.

$$\text{Recovery \%} = 100(\text{Conc}_{\text{found}}/\text{Conc}_{\text{theoretical}}) \quad \text{eq. 3}$$

where: $\text{Conc}_{\text{found}}$ represents the concentration as calculated from the regression equation, and $\text{Conc}_{\text{theoretical}}$ is the used concentration.

1.7.4. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

For the evaluation of LOD and LOQ we used the approach based on the standard deviation of the response (σ), calculated as standard deviation of Y-intercept, and the slope of the calibration curve (S) (ICH 2005) using equations 1 and 2:

$$\text{LOD} = (3.3\sigma)/S \quad (\text{eq. 1})$$

$$\text{LOQ} = (10\sigma)/S \quad (\text{eq. 2})$$

where 3.3 and 10 are constants, σ is the standard deviation of Y-intercept and S is slope of calibration curve.

Figure S1: NP-HPLC profile of the extracts. a) ISO; b) SE; c) SLDE 1:100; d) CM. **1:** (2E,4E,12Z)-N-isobutyloctadeca-2,4,12-trienamide and N-isobutyl-2E,4E-octadienamide; **2:** 2E,4E,14Z-N-isobutyleicosa-2,4,14-trienamide; **3:** retrofractamide A; **4:** piperonaline; **5:** piperanine; **6:** piperine.

Table S1. Extractive yield (%) and piperine content in different extracts of *P. longum*^a

Extraction method	Yield (%)	Piperine content (mg/g of extract)
ISO	12.8 ± 5.03	231.85 ± 0.21
CM	7.6 ± 3.72	143.60 ± 0.54
SE	8.7 ± 2.78	233.67 ± 0.12
SLDE-Naviglio 1:100 ^b	7.9 ± 1.51	277.78 ± 0.10
SLDE-Naviglio 1:50 ^b	6.3 ± 2.14	317.72 ± 0.18
SLDE-Naviglio 1:10 ^b	6.1 ± 2.60	191.62 ± 0.15

^aData are the means of three extractions ± SD. ^bg of fruits: ml of solvent used for the extraction.

Table S2. Regression equation, correlation coefficient of the linear calibration graphs (r^2), limits of detection (LOD), and limits of quantitation (LOQ) for the evaluation of piperine content in prepared extracts

Regression equation ^a ($y=ax+b$)	$y = 0.0557x + 0.0566$
Correlation coefficient (r^2)	0.9989
LOD	0.407
LOQ	1.232

^aIn the regression equation $Y = ax + b$, x denotes the concentration of the compounds ($\mu\text{g/mL}$), y is the absorbance, a is the slope and b is the intercept of the regression line.

Table S3. Precision and accuracy results.

Concentration ($\mu\text{g/mL}$)	Observed concentration ^{a,b} ($\mu\text{g/mL}$)	Precision ^c RSD%	Accuracy ^d RE%
4	4.27±0.123	2.95	106.80
6	6.49±0.13	2.04	108.20
8	8.14±0.15	1.88	101.70

^aCalculated on three different analyses; ^bmean±SD; ^cRSD%=(SD/mean)*100;

^dRE%=(mean found/real concentration)*100

Table S4. Intraday and interday precision.

Concentration (µg/mL)	Intraday		Interday precision	
	Observed	RSD% ^c	Observed	RSD% ^c
	concentration (µg/mL) ^{a,b}		concentration (µg/mL) ^{a,b}	
4	4.25±0.06	1.37	4.36±0.07	1.50
6	5.80±0.13	2.19	5.94±0.09	1.53
8	8.23±0.11	1.32	8.09±0.12	1.47

^aCalculated on six different analyses; ^bmean±SD; ^cRSD%=(SD/mean)*100