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Short communication

# HPLC method for the quantitative determination of sarcophine, a source of cembranoids with cancer chemopreventive activity

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**Abstract**

An accurate, reproducible and sensitive method for the quantitative determination of sarcophine in the organic extract of the Red Sea soft coral *Sarcophyton* species was developed and validated. Sarcophine concentration was determined by RP HPLC using ODS column. The mobile phase was made up of 70% acetonitrile in deionized water and the pH was adjusted to 3.5 with phosphoric acid. The flow rate was 1.5 ml/min and the detector was set to 220 nm. The HPLC analysis of several *Sarcophyton glaucum* samples collected from different locations in the Red Sea revealed that Hurghada had the highest sarcophine concentration.


*Keywords:* *Sarcophyton*; Cembranolide; Sarcophine; High performance liquid chromatography; Validation

## 1. Introduction

Sarcophine is a cembranolide diterpene isolated from the Red Sea soft coral, *Sarcophyton glaucum* order Alcyonaria and family Alcyoniidae (Fig. 1) [1,2]. It has toxic effects on fish, mice and rats. Sarcophine constitutes the corals chemical defense mechanism against natural predators [3]. It acts as an inhibitor of a number of vital enzymes including cholinesterase and phosphofructokinase by reacting with their thiol groups [3,4]. Sarcophine was also found to have a potential to inhibit tumorigensis assessed by its ability to inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced transformation [5]. The microbial transformation of sarcophine and its chemical oxidation with selenium dioxide yielded a group of compounds with cancer chemopreventive activity higher than sarcophine [5–7], some of which were recently patented [8].

Sarcophine is an unusual natural product in the sense that reported yields ranged from 1 to 3 % in dry weight of the *Sarcophyton* soft coral [3]. The high yield of sarcophine coupled with the significant cancer chemopreventive activity of its derivatives make this compound of special importance for drug discovery and development. However, the concentration of sarcophine in the coral is affected by location and season of collection [9]. Therefore, it is necessary to develop a rapid, accurate and valid method for the quantitative determination of sarcophine in the *Sarcophyton* extract.

## 2. Experimental

### 2.1. General

HPLC Shimadzu instrument, equipped with a model series LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12 A Degasser, Rheodyne 7725 injector with a 20 μl loop and a SPD-10AVP UV–vis detector and with a HPLC column TSK-Gel ODS-80 TM column, 5 μm, with dimensions 150 mm × 4.6 mm were used to determine the quantity of sarcophine in the organic extract. The UV spectral analysis of sarcophine was measured in double-beam Shimadzu UV–visible spectrophotometer, model

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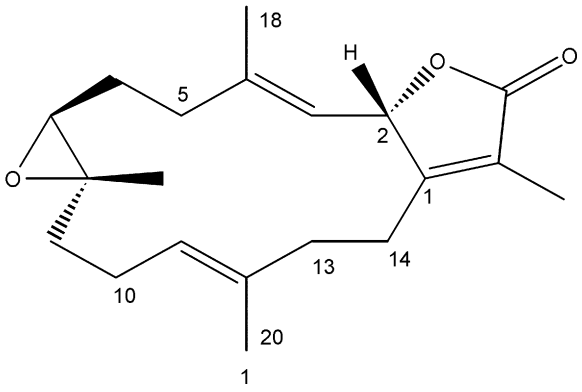


Fig. 1. Molecular structure of sarcophine isolated from *Sarcophyton glaucum*.

UV-1601 PC equipped with 1 cm quartz cells. The bundled software was UVPC personal spectroscopy software version 3.7. EM science silica gel 60/230–400 mesh (flash) was used for column chromatography. All reagents used were HPLC grade.

### 2.2. Animal material

Samples of the soft coral *Sarcophyton* species were collected by hand using scuba from different locations in the Red Sea: El-Gouna (GN), Hurghada (HU), Marsa Alam (MA), Ras Mohammed (RM), Safaga (SA) and Sharm El-Sheikh (SH). Samples were collected during October/November 2003. The samples were frozen immediately after collection in liquid nitrogen then at −80 °C until manipulation. Different *Sarcophyton* species were identified by the sclerite examination according to published keys [10].

### 2.3. Extraction

The frozen samples were freeze dried and then were extracted with methanol-dichloromethane mixture (1:1, v/v) for 5 days. The solvent mixture was replaced everyday to ensure extraction efficiency. The extract was dried under vacuum, dissolved in chloroform and partitioned against water to remove the sodium chloride traces from the extract then dried under vacuum. Complete extraction was confirmed by thin layer chromatography and high performance liquid chromatography.

Pure standard sarcophine was prepared by the fractionation of a portion of the organic extract of one sample on silica gel column using 30% ethyl acetate in hexane as an eluent. The fraction containing sarcophine was further purified by crystallization out of the cold hexane. The identity of sarcophine was confirmed by comparing the spectral data with the published data [1].

### 2.4. Chromatographic conditions

The best separation was obtained using a mobile phase acetonitrile–deionized water (70:30, v/v) and pH adjusted to 3.5 by phosphoric acid. The detector was set to 220 nm, the flow rate was 1.5 ml/min and injection volume was 20 μl. All determinations were performed at ambient temperature.

### 2.5. Standard solution and linearity

Six standard solutions were prepared by diluting the stock with methanol to reach a concentration range of 10–80 μg $ml^{-1}$. The used concentrations were 10, 20, 40,50, 60 and 80 μg $ml^{-1}$. The standard solution was filtered through 0.45 μm disposable filter. Triplicate 20 μl injections were chromatographed under the conditions described above. The peak area of each concentration was plotted against the corresponding concentration to obtain the calibration graph.

### 2.6. Sample solution

Samples were prepared by dissolving a specified weight of the extract in methanol to a final concentration of 20 mg% and filtered through 0.45 μm filters, consequently, 20 μl injection of each sample was assayed.

### 2.7. Statistical analysis

The means of dry weight concentrations of sarcophine in different species and from different locations were compared using one way analysis of variance (ANOVA). Significant results were determined as $P < 0.05$.

## 3. Results and discussion

The developed reversed phase high performance liquid chromatography (RP HPLC) method was applied to determine the sarcophine concentration in the organic extract. The chromatographic conditions were studied and optimized as a function of the pH and the acetonitrile concentration in the mobile phase. Fig. 2 shows the chromatogram of 40 μg/ml of standard sarcophine. The average retention time ± standard deviation (S.D.) was found to be 4.6 ± 0.012 min. The HPLC method was specific to sarcophine, hence we used short UV (220 nm) at which only sarcophine had maximal absorbance with minimal inter-

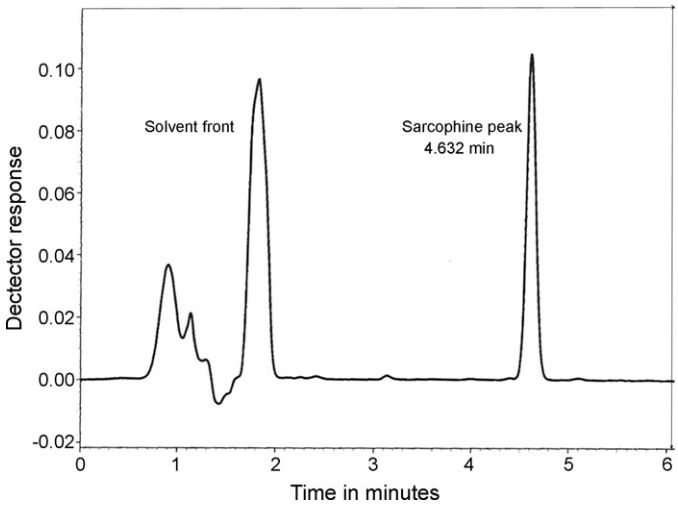


Fig. 2. A typical chromatogram of 20 μl injection of 40 μg/ml of sarcophine measured at 220 nm.

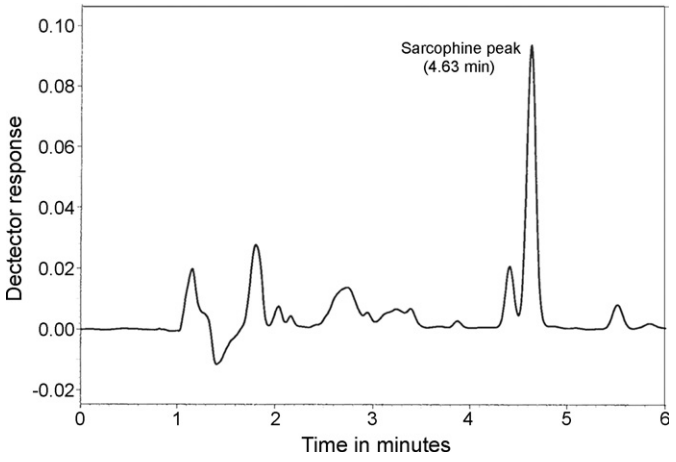


Fig. 3. Chromatogram showing the chromatographic separation of sarcophine from other organic compounds of *S. glaucum* from Marsa Alam. The sarcophine concentration was 2.3% of the dry weight.

ferences from other compounds in the extract. The specificity of the RP HPLC is illustrated in Fig. 3 where there is a good separation of sarcophine from the rest of the analyzed matrix.

### 3.1. Validation of HPLC method

#### 3.1.1. Linearity

The linearity of the HPLC method used in this study was evaluated by analyzing six concentrations of sarcophine 10, 20, 40, 50, 60 and 80 μg/ml in triplicates. The assay was performed according to the experimental conditions previously established. The linearity of the calibration graphs and adherence of the system to Beer's law were validated by the high value of correlation coefficient.

#### 3.1.2. Precision

For evaluation of the precision and the repeatability, three concentrations of the authentic sarcophine were assayed over several days. There was no significant difference in the mean results obtained from 1 day to another.

#### 3.1.3. Range

The calibration range was established to give accurate, precise and linear results. The calibration range was from 10 to 80 μg/ml (Table 1).

#### 3.1.4. Detection and quantitation limit

An approach based on standard deviation and slope of the standard curve was used to determine the detection limit. Also the same approach was used to determine the quantitation limit (Table 1).

#### 3.1.5. Selectivity

The HPLC method was selective for sarcophine. It was able to detect sarcophine in the complex natural extract without interference from other compounds in the extract.

Table 1
Validation parameters of the HPLC method

| Parameter | Value |
|---|---|
| Calibration range (μg/ml) | 10–80 |
| Detection limit (μg/ml) | 0.053 |
| Quantitation limit (μg/ml) | 0.17 |
| Regression equation ($Y$)[a] | $Y = 7196.5 + 17859.1C$ |
| Slope ($b$) | 17859.1 |
| Intercept ($a$) | 7196.5 |
| Standard deviation of the slope | 383.95 |
| Confidence limit of the slope[b] | 17423.9–18294.32 |
| Confidence limit of the intercept | −121854 to 26578.36 |
| Correlation coefficient | 0.99984 |
| Standard error of the estimation | 8703 |

[a] $Y = a + bC$, where $C$ is the sarcophine concentration in (μg/ml) and $Y$ is Peak area.
[b] 95% Confidence limit.

Peak purity was confirmed by collection of the eluted peak of sarcophine followed by comparing nuclear magnetic resonance spectra of the eluted peak with that of pure standard.

#### 3.1.6. Accuracy

The study was performed by the addition of known amounts of the authentic sarcophine to a sample with a known amount of sarcophine. The mixture was assayed and results were obtained and compared to the expected values. The percent recovery was 101.4%. This suggests acceptable accuracy of the HPLC method.

#### 3.1.7. Robustness

Variation of the pH of the HPLC mobile phase by ±0.1 and its organic strength by ±1.5% did not show significant effect on HPLC chromatographic resolution.

#### 3.1.8. Stability

*Sarcophyton* extract and the authentic sarcophine dissolved in methanol were stable when stored at 4 °C for up to 1 week without degradation.

#### 3.1.9. Statistical analysis

Comparison of dry weight concentrations of sarcophine among the different *Sarcophyton* species revealed that the concentration of sarcophine is significantly different from one species to another ($P = 0.014$) (Table 2a). *S. glaucum* was the highest sarcophine containing species. Moreover, Hurghada was

Table 2a
Percentage of sarcophine in the dry weight of the Red Sea soft coral *Sarcophyton* species

| Species | Sarcophine in dry weight (%) |
|---|---|
| *Sarcophyton auritum* ($n = 4$) | 0.05 ± 0.0041[*] |
| *Sarcophyton ehrenbergi* ($n = 3$) | 0.127 ± 0.018 |
| *Sarcophyton glaucum* ($n = 6$) | 8.238 ± 0.125 |
| *Sarcophyton trochliophorum* ($n = 4$) | 0.117 ± 0.017 |

[*] Mean ± S.E. Comparison among the groups was made using one-way analysis of variance.

Table 2b
Concentration of sarcophine in *S. glaucum* from different locations

| Location | Sarcophine in dry weight (%) |
|---|---|
| Sharm El-Sheik (SH) | 2.333 ± 0.186* ($n = 3$) |
| Hurghada (HU) | 8.283 ± 0.125($n = 6$) |
| Safaga (SA) | 3.717 ± 0.044($n = 3$) |
| Marsa Alam (MA) | 2.250 ± 0.029($n = 3$) |
| El-Gona (GN) | 1.467 ± 0.260($n = 3$) |
| Ras Mohammed (RM) | 4.253 ± 0.187($n = 3$) |

* Mean ± S.E. Comparison among the groups was made using one-way analysis of variance.

shown to be the location with the highest sarcophine concentration for the different *S. glaucum* samples (Table 2b).

## 4. Conclusion

To the authors' knowledge, this is the first report on an HPLC method for the quantitative analysis of sarcophine. The HPLC method described above provides a simple, accurate and reproducible quantitative analysis for the determination of sarcophine in *Sarcophyton* extracts without interference from other compounds in the extract.

This is also the first study that screens different *Sarcophyton* species from different locations in the Red Sea for the presence and quantity of sarcophine. Our results indicated that all *Sarcophyton* species produced sarcophine but with variable concentrations. *S. glaucum* was shown to produce the highest amounts of sarcophine. Our findings are consistent with the notion that sarcophine is a natural chemical defense for *Sarcophyton* against natural predators and space competitors [6]. Therefore, it was logical to find that all species produce sarcophine. This finding explains the low rate of mortality in *Sarcophyton* colonies and their long term stability despite having slow growth rates and being poor colonizers [11]. Furthermore, our results revealed that the sarcophine concentration is variable among the different species and within the individual species collected from different locations. This agrees with another report [12] which studied the distribution of sarcophytol A in the genus *Sarcophyton*. This difference may be attributed to the environmental conditions at the collection site, the presence of predators and/or stressful conditions that encourage the coral to produce more sarcophine as a chemical defense [12].

The proposed method serves as an effective approach to identify the S*arcophyton* species, location and appropriate environmental and/or aquaculture conditions for the production of highest yields of sarcophine. Sarcophine, once produced in large quantities can serve as a prototype for the production of numerous analogues that can later be tested for cancer chemopreventive activity [6].

Of the Red Sea species and locations tested using the described HPLC method; *S. glaucum* from Hurghada location produced the highest concentrations of sarcophine (Table 2a). Further studies are required to determine optimal mariculture conditions for highest yields of sarcophine.

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