

# Determination of oxidized scytonemin in *Nostoc commune* Vauch cultured on different conditions by high performance liquid chromatography coupled with triple quadrupole mass spectrometry

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**Abstract** A facile method based on high performance liquid chromatography coupled with electrospray ionization tandem triple quadrupole mass spectrometry (HPLC-ESI-QqQ-MS) has been established to investigate the production of the oxidized scytonemin by *Nostoc commune* Vauch in different environmental conditions. In optimized HPLC-ESI-QqQ-MS conditions, the oxidized scytonemin can be effectively detected in selected reaction monitoring (SRM) mode. After regression, high linearity of the calibration curve was achieved with a correlation coefficient ( $r^2$ ) at 0.99. The limit of detection and limit of quantification were 0.03 and 0.10 ng mL<sup>-1</sup>, respectively. The recovery of the oxidized scytonemin was at 76.90 %, and the relative standard deviations of inter- and intraday precisions were lower than 8.95 % ( $n=4$ ). Subsequently, the production of the oxidized scytonemin from *N. commune* has been investigated in different culture conditions. High culture temperature, strong illumination intensity, and light–dark cycle (12:12 h) were found to be good for producing the oxidized scytonemin by *N. commune*. In short, the HPLC-QqQ-SRM-MS method is a powerful tool for comprehensive analysis of the oxidized scytonemin from *N. commune*, owing to its excellent selectivity and sensitivity.

**Keywords** HPLC-MS · Oxidized scytonemin · *Nostoc commune* · Quantitative analysis

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## Introduction

Photosynthetic cyanobacteria have evolved a variety of strategies to prevent the damages caused by exposure to solar UV radiation, including DNA repair processes, UV avoidance behavior, and the synthesis of radiation-absorbing pigments (Balskus and Walsh 2008, 2010; Bebout and Garcia-Pichel 1995; Castenholz et al. 2000; Cockell and Knowland 1999; Gao and Garcia-Pichel 2011; Levine and Thiel 1987). UV-absorbing/screening pigments were first found in terrestrial cyanobacteria. The cyanobacterium, *Nostoc commune* Vauch, can retain viability for over 100 years during desiccation (Cameron 1962; Lipman 1941). As the water content decreases during desiccation, photosynthetic activity decreases, and no photosynthetic activity is detected in desiccated colonies (Sakamoto et al. 2009). This cessation of photosynthetic electron transport during desiccation is thought to be a protective response to avoid light-induced damage by suppressing reactive oxygen species production (Fukuda et al. 2008; Hirai et al. 2004). However, photosynthetic activity of the cyanobacterium recovers rapidly upon rehydration (Tamaru et al. 2005; Sakamoto et al. 2009; Satoh et al. 2002). With the UV-absorbing pigment of scytonemin in its extracellular matrices, *N. commune* is thought to be able to adapt to terrestrial environments with high levels of solar radiation (Matsui et al. 2011).

Scytonemin, a hydrophobic yellow-brown pigment, absorbs UV-A radiations from 320 to 400 nm and is found exclusively in the cyanobacterial sheath of around 300 cyanobacterial species (Geitler 1932; Desikachary 1959; Sinha and Häder 2008). It has been reported that the amount of scytonemin in cyanobacterial sheaths may be sufficient to prevent about 85–90 % of UV-A radiation from entering the cells (Garcia-Pichel and Castenholz 1991; Garcia-Pichel et al.

**Table 1** The components of medium BG11-C

Compound	Amount (g L <sup>-1</sup> )
NaNO <sub>3</sub>	1.5
K <sub>2</sub> HPO <sub>4</sub>	0.08
MgSO <sub>4</sub> ·7 H <sub>2</sub> O	0.075
CaCl <sub>2</sub>	0.02
Citric acid	0.006
Ferric ammonium citrate	0.006
Na <sub>2</sub> CO <sub>3</sub>	0.02
NaSiO <sub>4</sub> ·9H <sub>2</sub> O	0.025
EDTA	0.001
Trace metal mix A <sub>5</sub>	1.5

1992). Scytonemin is thought to be synthesized from metabolites of aromatic amino acid biosynthesis and can be induced by high photon fluence rate, and scytonemin can protect cyanobacteria when other ultraviolet-protective mechanisms, such as active repair of damaged cellular components, are ineffective.

Various analytical techniques have been widely used to study cyanobacteria and scytonemin; Raman spectrometry (Edwards et al. 1999), UV absorbance (Garcia-Pichel and Castenholz 1991; Dillon and Castenholz 1999), nuclear magnetic resonance (Proteau et al. 1993), HPLC, and mass spectrometry (MS) (Sinha et al. 1998; Squier et al. 2004a, b; Hunsucker et al. 2001; Jones et al. 2011) have been used to qualitatively or quantitatively analyze scytonemin. However, to our knowledge, a method based on high performance liquid chromatography tandem triple quadrupole coupled with selected reaction monitoring mass spectrometry (HPLC-QqQ-SRM-MS) to study the production of scytonemin by *N. commune* in different conditions has not been reported. HPLC-MS is a widely used technique for both qualitative and quantitative analyses of ingredients, which combines the efficient separation capability of HPLC with powerful structural characterization of MS. Moreover, the use of MS in the SRM mode results in high selectivity, as well as improved sensitivity compared to UV and evaporative light-scattering detection.

In this study, we developed a novel HPLC-QqQ-SRM-MS method for separation and determination of trace amount of the oxidized scytonemin in *N. commune*. Different culture conditions, such as irradiance, culture temperature, and light–dark cycle, were used to investigate the production of the oxidized scytonemin by *N. commune*.

## Materials and methods

The reference compound, oxidized scytonemin (91 %), was obtained from Merck (Germany). HPLC-MS grade dimethyl

sulfoxide (DMSO) and acetonitrile were supplied by Merck, and analytical grade trifluoroacetic acid was obtained from Sigma-Aldrich. Deionized water from a Milli-Q system (Millipore, USA) was used in all procedures.

The HPLC-MS analysis was carried out in Finnigan Surveyor and TSQ Quantum Access system (Thermo Fisher Scientific, USA) equipped with electrospray ionization (ESI) interfaced triple quadrupole mass spectrometer. All the operations, the acquiring and analysis of data, were processed by Xcalibur (Thermo Fisher Scientific).

## HPLC-MS conditions

In all cases, analyses were carried out at 30 °C on a reversed phase Hypersil Gold C18 (100×2.1 mm, 1.7 μm); A linear gradient elution of 0.1 % trifluoroacetic acid (A) and acetonitrile (B) was used with the gradient procedure as follows: 0–8 min, 10 to 50 % B; 8–15 min, 50–80 % B; 15–20 min, 80–90 % B; 20–25 min, 90–100 % B. The flow rate was 0.3 mL min<sup>-1</sup>, and the injection volume was 10 μL. The ionization conditions were adjusted as follows: sheath gas pressure (N<sub>2</sub>) flow rate, 30 L min<sup>-1</sup>; aux gas pressure (N<sub>2</sub>) flow rate, 10 arb; spray voltage, 2.8 KV; vaporizer temperature, 300 °C; and capillary temperature, 350 °C; argon was introduced into the trap with an estimated pressure 6×10<sup>-6</sup> mbar to improve trapping efficiency to act as the collision gas for the selected reaction mode data, and collision gas pressure was set at 1.5 mTorr. The HPLC-MS was operated in the positive ionization mode with data acquisition mode of SRM for quantification. The transitions monitored of the oxidized scytonemin in SRM mode were *m/z* 545.1 to 517.2 (28 V) and *m/z* 545.1 to 488.8 (29 V). Both product ions at *m/z* 517.2 and 488.8 were utilized as quantitative ions.

## Materials and sample preparation

*Nostoc commune* was collected from sandy soil in Ningbo University. Fresh *N. commune* was cleaned and then soaked in 75 % ethanol for 40 s. Subsequently, the *N. commune* was immersed in a combination of ampicillin (300 μg mL<sup>-1</sup>), kanamycin (100 μg mL<sup>-1</sup>), and gentamicin (100 μg mL<sup>-1</sup>) for 2 h. Finally, the *N. commune* was irradiated under UV light for 20 min.

The treated *N. commune* (0.2 g) was weighted and transferred into a sterile flask with 150-mL BG11-C liquid nutrient medium (shown in Table 1). Then, the flask was placed on a shaker and shaking at 35 rpm for 7 days. The culture conditions, such as illumination intensity, culture temperature, and light–dark cycle, were used to culture *N. commune*. The culture conditions are shown in Table 2.

After culture, the samples were dried at 37 °C for 24 h. Then, they were soaked and ground in acetone at 4 °C for 12 h. After extraction of the oxidized scytonemin from the treated samples by a 75 % acetone water solution under ultrasonication for

10 min, the acetone water solution with the oxidized scytonemin was separated by centrifugation at 10,000 rpm for 5 min. The extraction and centrifuge procedures were repeated for several times until the extract became colorless. After evaporation of the solvent, the concentrate was dissolved in 1.5 mL acetone, membrane filtered (0.45  $\mu\text{m}$ ), and analyzed by HPLC-MS.

#### Standard and solution preparation

To prepare stock solutions, the oxidized scytonemin was dissolved in the mixture of methanol and DMSO (1:1) to give a concentration of 1 mg mL<sup>-1</sup>. Working solutions were obtained by dilution of the stock solution with the mixture of methanol and DMSO (1:1).

The recovery rate (in percent) was calculated as the ratio of the peak area for a spiked amount of the oxidized scytonemin standard (10  $\mu\text{g mL}^{-1}$ , 50  $\mu\text{L}$ ) in *N. commune* that contained no detectable oxidized scytonemin passing through the sample preparation procedure to the peak area for the same amount of the spiked standard in a neat solution, as analyzed by HPLC-QqQ-SRM-MS.

In order to evaluate the ion suppression of the complexity samples, the experiment has been achieved as following: the 0.1 g *N. commune* with additional known amount of oxidized scytonemin (10  $\mu\text{g mL}^{-1}$ , 1 mL) was used as spiked sample, and original sample was obtained from the extract of 0.1 g *N. commune*. Both spiked and original samples were enriched and purified according to the sample preparation procedure. Then, the spiked and original samples were both analyzed by HPLC-QqQ-SRM-MS.

## Results and discussion

#### Optimization of SRM/MS conditions

HPLC-QqQ-SRM-MS was performed to analyze the oxidized scytonemin in *N. commune*. According to the retention times

and mass spectra of the corresponding standard, oxidized scytonemin ( $t_R$  13.8 min) and reduced scytonemin ( $t_R$  12.8 min) were identified in HPLC-ESI-QqQ-MS from the protonated molecules ( $m/z$  545 and 547, respectively). In this paper, only oxidized scytonemin was analyzed by HPLC-QqQ-SRM-MS, and the SRM mode provided better sensitivity than the full scan mode. The results indicated that the SRM mode had more advantages in the quantification of low content analysis and separating overlapped constituents in complicated mixtures. Figure 1 shows the total ionization chromatogram (TIC) and SRM chromatogram of the extract of *N. commune*.

In the majority of MS<sup>2</sup> scan taken across the scytonemin peak, the ions at  $m/z$  517.2 and 488.8 were generated by neutral losses of 28 and 56 Da, which were assigned to elimination of one and two CO from cyclopentyl carbonyl groups, respectively (Fig. 2). These two fragment ions were the base peak and had the high relative abundances in MS<sup>2</sup> spectrum, thus they were chosen to be the fragment ions at SRM mode. The transitions monitored of the oxidized scytonemin in SRM mode were  $m/z$  545.1 to 517.2 (28 V) and  $m/z$  545.1 to 488.8 (29 V).

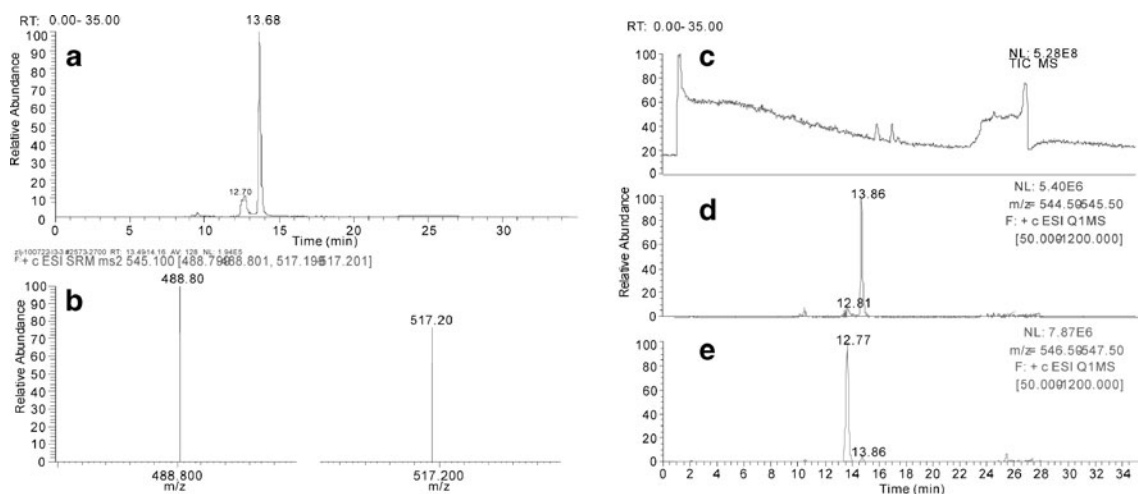
#### Method validation

Reliable and robust quantitative analyses utilizing HPLC-QqQ-SRM-MS were achieved with the oxidized scytonemin standard solutions ranging from 0.5, 1, 5, 10, 25, and 50  $\mu\text{g mL}^{-1}$ . The calibration curve of the oxidized scytonemin showed good linear regression with an  $R^2=0.99$  within relatively wide test ranges. Table 3 summarizes the result of the calibration curve of scytonemin.

The relative standard deviations (RSDs) evaluated with the oxidized scytonemin were taken once a day and tested for five consecutive days. The RSDs for the intra- and interday precisions were achieved in three different concentrations (0.5, 5, and 25  $\mu\text{g mL}^{-1}$ ). The area peak reproducibility ( $A_r$ ) was acceptable with RSD values ranging from 1.48 to 4.46 % for intraday precision and 4.19 to 8.95 % for

**Table 2** The contents of the oxidized scytonemin in different culture conditions

Analytes	Culture time (days)	Light–dark cycle (h)	Illumination intensity ( $\mu\text{mol protons m}^{-2}\text{s}^{-1}$ )	Culture temperature ( $^{\circ}\text{C}$ )	Average content ( $\mu\text{g g}^{-1}$ , $n=2$ )
A	7	12:12	25	25	150.2 $\pm$ 6.5
B	7	12:12	25	30	178.9 $\pm$ 26.6
C	7	12:12	25	35	271.7 $\pm$ 13.5
D	7	12:12	50	25	210.1 $\pm$ 8.2
E	7	12:12	50	30	509.5 $\pm$ 22.3
F	7	12:12	50	35	758.1 $\pm$ 34.0
G	7	24:0	50	25	189.6 $\pm$ 16.2
H	7	24:0	50	30	359.3 $\pm$ 4.0
I	7	24:0	50	35	334.8 $\pm$ 25.8



**Fig. 1** The total ionization chromatogram and SRM chromatogram of the extract of *N. commune*. **a** The SRM chromatogram of the oxidized scytonemin in *N. commune*. **b** The transitions monitored of the oxidized scytonemin in SRM mode. **c** The TIC chromatogram of the

extract of *N. commune*. **d** The extract ion chromatogram of the oxidized scytonemin. **e** The extract ion chromatogram of the reduced scytonemin

interday precision by analyses of HPLC-QqQ-SRM-MS. The data suggested that the method showed good reproducibility in terms of peak area. The results from the quantitative analysis of the precision of this method could achieve the desired requirements.

The limit of detection (LOD) and limit of quantification (LOQ) values were determined by first dilution to 100 ng mL<sup>-1</sup> standard solution, and an aliquot of 10 μL was injected. The peak signal to noise ratio (S/N in accordance with 3/1 for LOD and 10/1 for LOQ) was obtained. The results demonstrated that the method was very sensitive with LOD (0.03 ng mL<sup>-1</sup>) and LOQ (0.11 ng mL<sup>-1</sup>) for the oxidized scytonemin.

The recovery was used to evaluate the accuracy of the method. The *N. commune* without oxidized scytonemin was used as blank matrix spiking with known amounts of the standard (10 μg mL<sup>-1</sup>, 50 μL). Sample was enriched and purified according to the method in “Standard and solution preparation.” The recovery was obtained as 76.9 % (RSD=9.2 %, n=5) by the above method. The result showed that

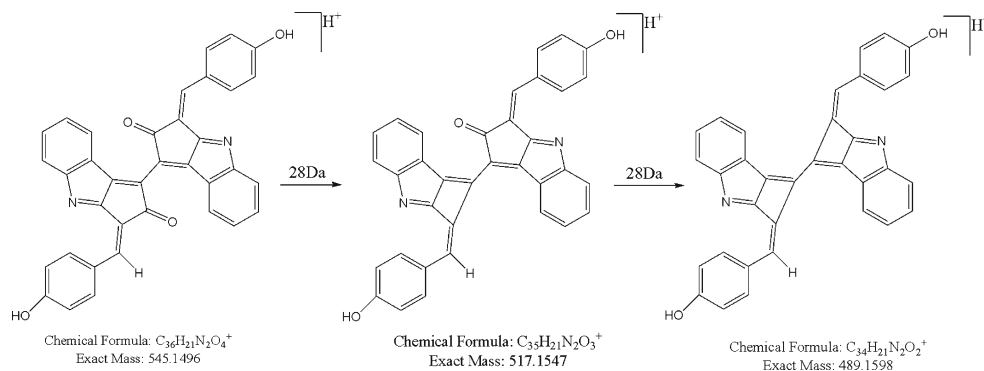
the method had acceptable accuracy and repeatability for the oxidized scytonemin.

The spiked and original samples for the ion suppression evaluation were both analyzed by HPLC-QqQ-SRM-MS. The results showed that the original sample contained 21.8 μg oxidized scytonemin, and in the spiked sample, the amount of the oxidized scytonemin was 32.7 μg close to the expected 31.8 μg oxidized scytonemin in the spiked sample, indicating that the ion suppression effect did not obviously affect the accuracy of the quantitative analysis.

The influence of culture condition in the oxidized scytonemin from *N. commune*

This study had the advantage of allowing comparison of several culture conditions using environmental samples rather than reconstructed lab samples, in order to assess organisms in their natural state, including adaptation to environmental conditions and also possibly damaged or slow replicating organisms. Different culture conditions,

**Fig. 2** The fragmentation pathways of the oxidized scytonemin



**Table 3** Calibration curve and their LOD, LOQ, precisions, and recovery of the oxidized scytonemin

Analyte	Calibration curve	$r^2$	Test range ( $\mu\text{g mL}^{-1}$ )	LOD ( $\text{ng mL}^{-1}$ )	LOQ ( $\text{ng mL}^{-1}$ )	Recovery ( $n=5$ ) (%)	Intraday precision (RSD%, $n=4$ )			Interday precision (RSD%, $n=4$ )		
							0.5 $\mu\text{g mL}^{-1}$	5 $\mu\text{g mL}^{-1}$	25 $\mu\text{g mL}^{-1}$	0.5 $\mu\text{g mL}^{-1}$	5 $\mu\text{g mL}^{-1}$	25 $\mu\text{g mL}^{-1}$
							Scytonemin	$Y=3,032,080 \times X+1,698,280$	0.9925	0.5–50	0.03	0.11

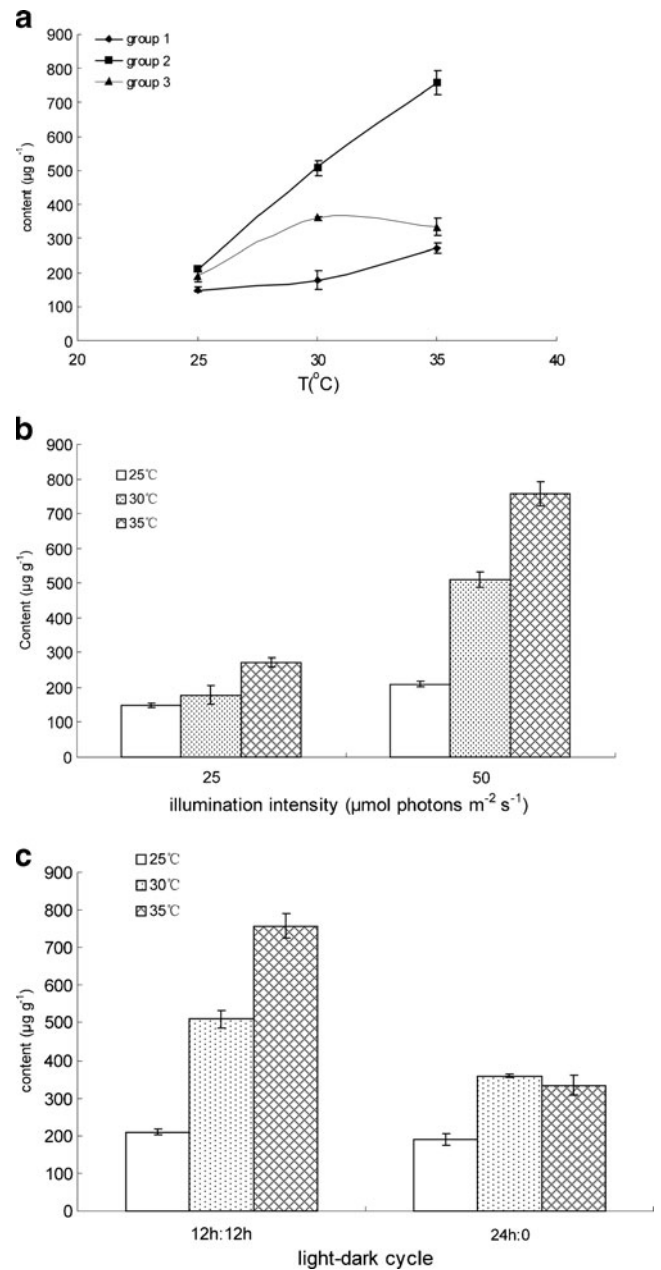
such as irradiance, culture temperature, and light–dark cycle, were studied. According to the culture condition, nine samples were divided into three groups. Group 1 (assigned as A, B, and C) was defined as the culture condition of a light–dark cycle of 12:12 h, irradiance 25  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ , and the culture temperature was changed from 25 to 35 °C, by steps of 5 °C. Group 2 (assigned as D, E, and F) was defined with a light–dark cycle of 12:12 h, with an irradiance of 50  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ , and the culture temperature was changed from 25, 30, to 35 °C. Group 3 (assigned as G, H, and I) was defined as a light–dark cycle of 24:0 h, with an irradiance of 50  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ , and the culture temperature was changed from 25 to 35 °C, by steps of 5 °C. All of the samples of groups 1 to 3 were obtained by sunlight irradiation for 7 days. The scytonemin in these three groups of samples with different culture conditions were identified by comparing the retention time, the online UV, and MS information with authentic standard. The oxidized scytonemin of three groups were simultaneously determined, and the amounts of the scytonemin in the samples are listed in Table 2. The results showed that there were significant differences in the contents of three classes of the oxidized scytonemin.

The influence of culture temperature

Figure 3a shows that the oxidized scytonemin content changed according to the different culture temperatures. In groups 1 and 2, the contents of the oxidized scytonemin increased when the temperature was changed from 25, 30, to 35 °C. However, in the samples of group 3 which was different with a 24:0-h light–dark cycle, the content of scytonemin increased when the culture temperature changed from 25 to 30 °C. As the culture temperature rises to 35 °C, the content of the oxidized scytonemin increased steadily. The data presented here clearly suggest that the accumulation of the oxidized scytonemin was correlated to the culture temperature increasing with increasing temperature.

The influence of irradiance

The irradiance also influenced in the amount of oxidized scytonemin (Fig. 3b). The samples of groups 1 and 2 were utilized, with the culture conditions differing only in the



**Fig. 3** The influence of culture temperature, illumination intensity, and light–dark cycle of the oxidized scytonemin. **a** The influence of culture temperature of scytonemin. **b** The influence of illumination intensity of scytonemin. **c** The influence of light–dark cycle of scytonemin

irradiance. The content of oxidized scytonemin increased with increasing irradiance. Furthermore, the higher the culture temperature, the larger the increment in the amount of oxidized scytonemin. According to these results, it can be suggested that, at high temperature and strong solar irradiation, *N. commune* would produce a number of UV-protecting pigments (the oxidized scytonemin) for adapting this environment.

#### The influence of light–dark cycle

The light–dark cycle also plays an important role in the stimulation of oxidized scytonemin in *N. commune* as shown in Fig. 3c. The samples of groups 2 and 3 were used, which only differed with respect to light–dark cycle. Here, the amount of oxidized scytonemin was reduced as the light–dark cycle was changed from 12:12 to 24:0 h. Furthermore, the decrease in the oxidized scytonemin content was enhanced at higher culture temperature. These results suggest that, in photosynthesis, the carbon cycle worked in coordination with light reaction in generating the UV pigment—scytonemin. Thus, the 12:12-h light–dark cycle stimulated the production of oxidized scytonemin.

In conclusion, a simple and rapid quantitative HPLC-QqQ-SRM-MS method was developed for the comprehensive analysis of the oxidized scytonemin in *N. commune*. According to this method, the amount of the oxidized scytonemin was definitely identified based on SRM mode. The HPLC-QqQ-SRM-MS method was also successfully applied to monitoring of the amount of the oxidized scytonemin in *N. commune* and screening the best culture conditions (temperature, light–dark cycle, and irradiance). The results presented here are intended to provide information for helping to monitor and investigate oxidized scytonemin from *N. commune*.

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