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Article

High-Performance Thin-Layer Chromatography for Quantification of 1-Octacosanol in Antarctic Krill (*Euphausia superba* Dana)

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1-Octacosanol is a straight-chain aliphatic 28-carbon fatty alcohol with well-known anti-fatigue function. In this study, 1-octacosanol was extracted from Antarctic krill for the first time. Separation of 1-octacosanol was achieved using high-performance thin-layer chromatography (TLC) with a mobile phase consisting of petroleum ether/ethyl acetate/toluene (4 : 1 : 0.05, v/v/v) on precoated silica gel GF_{254} high-performance TLC plates. The separated 1-octacosanol was quantified using spectrodensitometry with distilled water/bromothymol blue/sodium hydroxide (100 : 0.1 : 0.7, v/w/w) as a chromogenic system. The high-performance TLC method was validated with respect to specificity, linearity, intra- and interplate variation. The stability of the 1-octacosanol–chromogen complex and recovery of 1-octacosanol were also evaluated. Containing ~10.6 μg/mg 1-octacosanol, Antarctic krill is potentially a rich and renewable source of 1-octacosanol.

Introduction

1-Octacosanol, a long-chain aliphatic alcohol, is a compound with well-known anti-fatigue function (1). 1-Octacosanol also has antiangiogenic effect and inhibits matrix metalloproteinase (2, 3). It also possesses a wide range of health-promoting benefits and pharmacological activities, such as lowering cholesterol or blood lipid (4, 5), improving athletic performance (6), reducing platelet aggregation (7) and decreasing risk of ulcer (8). Furthermore, it was reported that 1-octacosanol could be used in developing new strategies to prevent pain and inflammation (9). Long-term clinical studies have demonstrated that 1-octacosanol is safe and well tolerated (10). Because of these beneficial biological activities and its high stability as a compound, 1-octacosanol has been widely used in healthy foods, medicine and cosmetics.

Many healthy foods and raw materials contain 1-octacosanol at different levels. It is important to establish a reliable and simple analytical method to quantify 1-octacosanol content in various samples. Different methods, such as gas chromatography (GC) and GC–mass spectrometry (GC–MS) (11–13), have been used in analysis of 1-octacosanol. However, 1-octacosanol needs to be chemically derivatized before it can be reliably analyzed using GC or GC–MS (14). The ultra-performance liquid chromatography coupled with evaporative light scattering detector method (14) does not need 1-octacosanol to be derivatized, but because repeated sample injections increase the background noise level, the detector needs to be cleaned frequently. Yet, the cleaning process is time consuming. Thin-layer chromatography (TLC) is a method commonly used for separation, purification, identification and/or quantification of many compounds (15–19). Recently, Shashidhara and Nandini (15) used high-performance TLC for quantification of 1-octacosanol in *Tinospora*. However, the reported retention factor (R_f) value was much higher than the upper limit of the R_f range, 0.3–0.7, needed for accurate TLC analysis. In this study, a new high-performance TLC method was developed for separation and quantification of 1-octacosanol. This TLC method was systematically evaluated with respect to its specificity, linearity, precision, sensitivity and accuracy. Its utility was demonstrated by analyzing 1-octasanol extracted from Antarctic krill, which distributes abundantly in the Scotia Sea, Antarctic Peninsula and Western Weddell Sea regions. This krill species probably has the largest biomass among the multicellular animal species on earth (20). So far, there is no report in the literature that shows the presence or extraction of 1-octacosanol from Antarctic krill. Our results demonstrate that Antarctic krill is a rich and renewable source of 1-octacosanol.

Experimental

Test sample, reagents and instrumentations

A sample of frozen whole Antarctic krill with moisture content ~80% was obtained from Shandong Keruier Biological Products Co., Ltd (Shandong, China). 1-Octacosanol standard (≥99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All the other chemicals used in the study were of analytical grade. The petroleum ether used in this study has a boiling temperature range from 60 to 90°C. Pre-coated silica gel GF_{254} high-performance TLC plates (10×10 cm^2) were purchased from Haiyang Chemical (Shandong, China). A TLC scanner 3 equipped with the Wincats 1.4.1 software was purchased from CAMAG (Muttenz, Switzerland). An FUD-1200 freeze dryer was purchased from Tokyo Rikakikai Co., Ltd (Tokyo, Japan). A rotavapor-3 rotating evaporator was purchased from Buchi (Flawil, Switzerland). A development chamber ($10 \times 12 \times 5$ cm^3, consisting of a twin-trough glass chamber) was purchased from Shanghai Xinyi Instrument Co., Ltd (Shanghai, China).

Preparation of sample solution

One hundred grams of frozen whole Antarctic krill were freeze-dried at −46°C, resulting in ~20 g dry Antarctic krill. The 20 g freeze-dried krill and 120 mL *n*-hexane were combined in a beaker and stirred for 1 h. The mixture was filtered through one layer of 100-mesh nylon cloth. The above extraction procedure was repeated three times. All the filtrates were combined and centrifuged at 10,000 rpm at room temperature (23 ± 2°C)

for 3 min. The supernatant was subjected to rotating evaporation at 25°C to remove the *n*-hexane solvent and recover Antarctic krill oil.

The extracted Antarctic krill oil (3 g) was placed in a round-bottom flask containing 120 mL 90% ethanol and 12 g powdered sodium hydroxide and then refluxed at 80°C for 2 h. After cooling down to 50°C, 120 mL petroleum ether was added and reflux continued for 40 min but at 70°C. Then, 120 mL distilled water was added and the content was stirred for 20 min at 50°C. The extraction procedures were repeated three times. After the mixture layered, the combined petroleum ether phase (the supernatant) was removed by rotating evaporation at 30°C. The residue that contained 1-octasanol was dissolved in 8 mL chloroform.

Preparation of standard solution

A standard solution of 1-octacosanol was prepared by dissolving 1.0 g of 1-octacosanol (≥99%) in 4 mL chloroform, resulting in a standard solution with a concentration of 0.25 mg/mL 1-octacosanol.

Process of high-performance TLC

High-performance TLC was performed on pre-coated silica gel GF_{254} high-performance TLC plates, which were prewashed with chloroform, air dried and activated by incubation at 110°C for 20 min to reduce background that can result from adsorption of volatile compounds from the environment. Antarctic krill 1-octacosanol sample solution and 1-octacosanol standard solution were separately spot applied to the plates, 1 cm apart and 1 cm from the left, right and bottom edges of the plates. After the spots were air dried at room temperature, the plates were developed in a twin-trough glass chamber ($10 \times 12 \times 5$ cm^3) that was previously saturated with vapor of the mobile phase for 20 min. The mobile phase was petroleum ether/ethyl acetate/toluene (4 : 1 : 0.05, v/v/v), and the development distance was 8 cm. After removed from the glass chamber, the plates were air dried at room temperature, flushed with the chromogenic system (distilled water/bromothymol blue/sodium hydroxide, 100 : 0.1 : 0.7, v/w/w) for 1 min, dried in a hot air oven (50°C) and then cooled at room temperature for 1 h. Densitometric scanning was performed with a Camag TLC scanner 3 at the reflectance–absorbance wavelength of 615 nm using a wolfram lamp. The scanning speed was 20 mm/s, and the data resolution was a 50-μm step. Peak areas were tracked in the entire detection. The densitometric data were analyzed using the Wincats 1.4.1 software.

Validation of the high-performance TLC method

Specificity

Standard solution of 1-octacosanol and Antarctic krill 1-octacosanol sample solution were each spotted on a high-performance TLC plate in two replicates. The process of high-performance TLC was the same as described previously. A spot of 1-octacosanol at $R_f = 0.45$ was found, and a peak purity test was also performed to determine the spectra of the spot. Chloroform was spotted in parallel as a negative control. To determine the impact of the chromogen staining on absorption curve, a blank high-performance TLC plate was included in parallel and processed as mentioned previously.

Linearity

Five different amounts of 1-octacosanol were each spotted on a high-performance TLC plate in triplicate: 375, 500, 625, 750 and 1,125 ng, corresponding to 1.5, 2.0, 2.5, 3.0 and 4.5 μL of the 1-octacosanol standard solution. Then, 1.5 μL of the Antarctic krill 1-octacosanol sample solution was also spotted in triplicate on the high-performance TLC plate. The process of high-performance TLC was performed as mentioned previously. The average peak values were calculated and used in linear regression between amount 1-octacosnal and peak area to determine the linearity of 1-octacosanol detection.

Intraplate variation and interplate variation

To evaluate precision and repeatability of the method, intra- and interplate variations were evaluated. To evaluate intraplate variation, the same volume (3.6 μL) of the 1-octacosanol standard solution was spotted on one high-performance TLC plate in five replicate spots. To determine interplate variation, the same volume (3.8 μL) of the standard solution was spotted on five plates in five replicate spots per plate. High-performance TLC was performed as described previously. All peak areas were measured and analyzed.

Stability

Stability of the 1-octacosnal–chromogen complex formed during staining process was determined by determining the peak areas of spots at different hours post plate staining. Briefly, three spots of the 1-octacosanol standard solution were spotted on one high-performance TLC plate. After the TLC plate was developed as mentioned previously, it was scanned 1, 2, 3, 4, 5, 6, 7 and 8 h after staining with the chromogen. All peak areas were measured and analyzed.

Recovery

Three aliquots of the 1-octacosanol sample solution prepared from Antarctic krill were spiked with three different amounts of the 1-octacosanol standard: 50, 100 and 150% of the amount of 1-octacosanol present in the Antarctic krill 1-octacosanol sample solution. The mixtures were then analyzed in triplicate as described previously.

Recovery was calculated as follows:

$$\text{Recovery } \% = \left[\frac{A - B}{C}\right] \times 100\%,$$

where A is both the quantity of 1-octacosanol originally present in the Antarctic krill 1-octacosanol sample and the quantity of 1-octacosanol spiked in from the standard, B is the quantity of 1-octacosanol only present in the Antarctic krill 1-octacosanol sample and C is the quantity of 1-octacosanol spiked from the 1-octacosanol standard solution.

Limit of detection and limit of quantification

In order to determine the limit of detection, the standard solution of 1-octacosnal was serially diluted to 20 μg/mL. Different amounts of the diluted 1-octacosanol were spotted on a high-performance TLC plates in 10 replicates. The limit of detection

was defined as the amount of 1-octacosanol that produced a 3 : 1 signal-to-noise ratio. The limit of quantification was determined by the amount of 1-octacosnal that produced a 10 : 1 signal-to-noise ratio.

Statistical analysis

Statistical analysis was carried out with SPSS 19.0 for Windows (SPSS, Chicago, IL, USA).

Caution

Toluene and chloroform are toxic chemicals. All of the steps using toluene or chloroform should be done in a fume hood, and researchers should wear anti-poison respirator. Spent reagents need to be collected and disposed of per relevant regulations and policies.

Results and discussion

Specificity

The specificity of the method was determined by analysis of the 1-octacosanol extracted from Antarctic krill as a sample, the standard solution of 1-octacosanol as a reference and chloroform as control. Spot 1 (Antarctic krill sample solution) and Spot 2 (standard solution) were aligned (Figure 1) at the R_f value of 0.45 (Figures 2 and 3). As shown in Figure 4, the absorption wavelength curve of the Antarctic krill 1-octacosanol sample was also the same as that of the 1-octacosanol standard. No interfering peak from the control was observed at the R_f value of 1-octacosanol. The chromogen, bromothymol blue, had no effect on absorption wavelength curve either. Based on these results, the method was deemed specific for 1-octacosanol.

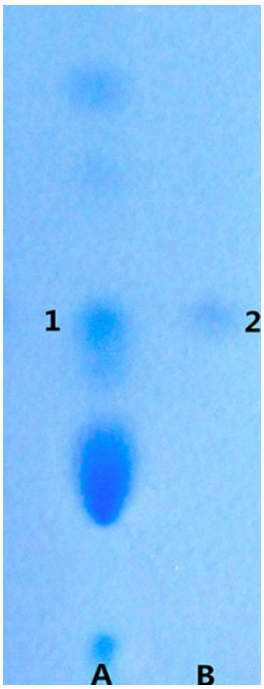

Figure 1. High-performance TLC plate after staining process. A: Antarctic krill sample solution and B: 1-octacosanol standard solution.

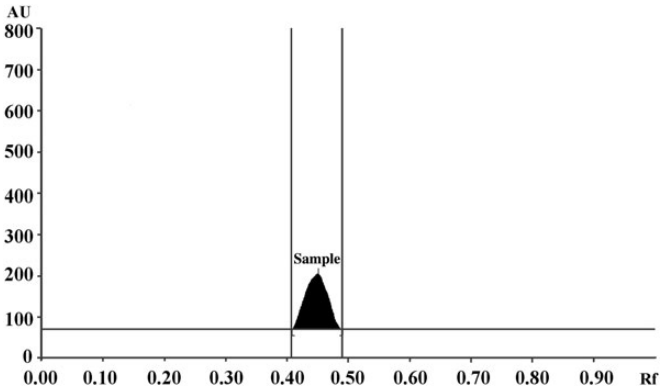

Figure 2. Peak of 1-octacosanol in Antarctic krill sample solution at $R_f = 0.45$.

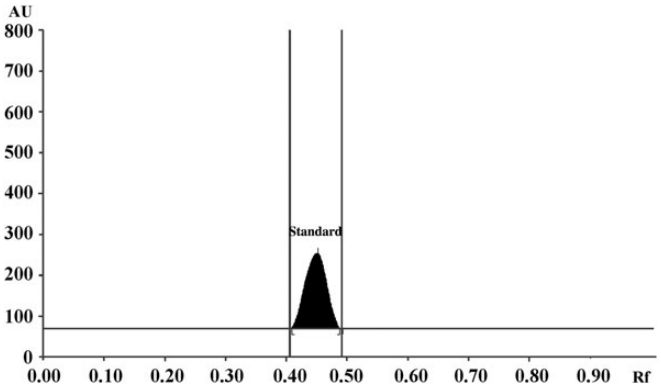

Figure 3. Peak of 1-octacosanol in standard solution at $R_f = 0.45$.

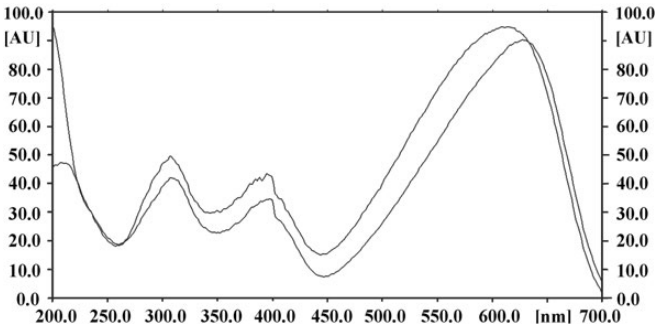

Figure 4. Spectrodensitometric analysis of 1-octacosanol obtained from standard 1-octacosanol and extract of Antarctic krill.

Linearity

The linear regression equation between amount of 1-octacosanol and peak area was

$$y = 20.5552 + 10.8837x (R^2 = 0.99962),$$

where y is the peak area and x is the amount of 1-octacosanol per spot. The relative standard deviation (RSD) was 1.35%. The linear range of 1-octacosanol detection was from 375 to 1,125 ng (Table I). Based on the above regression equation, the content of 1-octacosanol present in the Antarctic krill sample was

Table I
Average Peak Areas Corresponding to Different Amounts of 1-Octacosanol

Spot	Volume (μL)	1-Octacosanol (ng)	Average peak area (AU)
Standard 1	1.5	375.00	4,215.48
Standard 2	2.0	500.00	5,391.03
Standard 3	2.5	625.00	6,723.18
Standard 4	3.0	750.00	8,208.09
Standard 5	4.5	1,125.00	12,297.44
Sample	1.5	596.66	6,514.39

Table II
Intraplate Variation and Interplate Variation

Spot	Intraplate variation		Interplate variation	
	Peak area (AU)	RSD (%)	Peak area (AU)	RSD (%)
1	9,800.2		10,046.3	
2	9,792.5		10,371.6	
3	9,508.4	2.8102	10,515.2	2.6442
4	10,046.3		10,806.0	
5	10,238.4		10,360.0	

RSD, relative standard deviation.

Table III
Stability of 1-Octacosanol–Chromogen as Indicated by Peak Areas at Different Hours Post Staining Process

Time	Area of Spot 1 (AU)	Area of Spot 2 (AU)	Area of Spot 3 (AU)
1 h	11,446.9	9,674.8	11,186.1
2 h	11,447.6	9,653.2	11,314.6
3 h	11,528.5	9,720.6	11,340.4
4 h	11,522.7	9,715.9	11,302.0
5 h	11,540.7	9,767.6	11,300.7
6 h	11,354.3	9,697.6	11,040.6
7 h	10,648.2	8,912.1	10,395.4
8 h	10,293.8	8,904.0	10,454.8
RSD (1–5 h) (%)	0.4012	0.4569	0.5276
RSD (1–8 h) (%)	4.2543	3.8969	3.5598

RSD, relative standard deviation.

Table IV
Recovery Data

Level (%)	Recovery (%)	Average recovery (%)	RSD (%)
50	99.30		
100	99.17	99.12	0.2058
150	98.90		

RSD, relative standard deviation.

10.6 μg/g Antarctic krill wet biomass. As estimated, 70–200 megaton of Antarctic krill can be harvested annually (20). Based on the 1-octacosanol content determined in this study (10.6 μg/g), 742–2,120 tons of 1-octacosanol can be extracted from Antarctic krill annually.

Intraplate variation and interplate variation

The RSD for intraplate variation (based on peak area) was 2.8102%, while that for interplate variation was 2.6442% (Table II). These data suggest small variations within and between high-performance TLC plates. Thus, the new high-performance TLC method is precise and reliable for 1-octacosanol analysis.

Stability

Similar results were obtained at different hours (up to 5 h) after staining process (Table III). The 1-octacosanol–chromogen complex appeared to be stable at room temperature to allow completion of high-performance TLC process.

Recovery

On average, the high-performance TLC method allowed 99.12% recovery (Table IV) of the 1-octacosanol spiked in the Antarctic krill sample, again suggesting that the method is accurate for analysis of 1-octacosanol present in actual samples.

Limit of detection and limit of quantification

As performed in this study, the high-performance TLC method detected as little as 39 ng of 1-octacosanol (at a signal-to-noise ratio of 3 : 1). The limit of quantification for 1-octacosanol was determined to be 130 ng (at a signal-to-noise ratio of 10 : 1). These levels of sensitivity should allow detection and quantification of 1-octacosanol in many types of materials.

Conclusion

Compared with previous methods, the method developed in this study has several improvements. First, the high-performance TLC method allowed for not only separation and identification of 1-octacosanol but also quantification. Second, using a mobile phase consisting of petroleum ether/ethyl acetate/toluene (4 : 1 : 0.05, v/v/v), the R_f value (0.45) stayed near the middle of the R_f range (0.3–0.7) that is more reliable for TLC analysis. Additionally, bromothymol blue was used to visualize 1-octaco sanol on TLC plates for the first time. This chromogen allowed sensitive and linear detection of 1-octacosanol. Collectively, the validation showed that the new TLC method is a reliable method for 1-octacosanol analysis, including separation, identification and quantification. Although not demonstrated on other samples, the method may be used in analysis for 1-octacosanol from other types of samples.

In this study, 1-octacosanol was extracted from Antarctic krill for the first time. Therefore, Antarctic krill is potentially a rich and sustainable source of 1-octacosanol that warrants further study.

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