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A New HPLC-analytical Method for Total Sphingosine Contents as an Indirect Index for the Ganglioside Contents of Deer Antlers

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Abstract – Routinely applicable HPLC assay procedures for the ganglioside content in various deer antler preparations were established through the creation of a UV-absorbing chromophoric substance - trans-α,β-unsaturated-hexadecene-aldehyde - from the sphingosine moiety in ganglioside molecules by two step chemical reactions. In order to guarantee the assay's accuracy and sensitivity, the HPLC-assay procedure adopted internal reference procedures by mixing cis-α,β-unsaturated-hexadecene aldehyde[V] or cis-3-heptadecene- 1,2-diol[IV] to assay samples. The internal reference compound [IV] or [V] was synthesized in our laboratory starting from mannitol-diacetonide through three or four step organic reactions. This new HPLC-assay procedure was successfully applied to deer antler extracts with good dose-dependent calibration curves at the picomole level of gangliosides.

Keywords – HPLC-sphingosine, deer antler, ossified deer antler, α,β-unsaturated-alkene-aldehyde

Introduction

Quality evaluation of various preparations of deer antlers has been conducted through the analysis of general chemical components without referring to the biologically active components (Kim and Rhyu, 2000; Ha *et al*., 2003). Recently Ikeda *et al*., 1992 reported immuno-modulating activity of gangliosides and Fong *et al*., 1997 reported the memory enhancing activity of gangliosides. Hong *et al*. suggested using the gangliosides as index components in the quality evaluation of various preparations of deer antler by densitometry on thin-layer chromatograms (Hong *et al*., 1991, 1993; Han and Jhon, 1994). However TLC analytical procedures inevitably have greater limitations in the accuracy of analysis due to the poor resolution of TLC-spots. Techniques using HPLC and mass spectroscopy to assay gangliosides (Merrill Jr. *et al*., 2005; Sisu *et al*., 2011) were reported as was an HPLC method to analyze sphingolipids by derivatization with a fluorescent chromophore (Groener *et al*., 2007).

The present studies were conducted to establish a new HPLC assay procedure for the quantitative and routinely applicable analysis of total sphingosine content in place of gangliosides in deer antlers, considering that many sphingolipids are able to serve as resource molecules for the biosynthesis of gangliosides in our body when extracts of deer antlers are administered to the human body for the improvement of health-care. A ganglioside is one of the glycol-sphingo-lipids composed of fatty-acyl-amides of sphingosine-trioside. Micro-analysis of gangliosides or sphingosine by HPLC is very difficult due to the absence of UV-absorbing chromophores in the molecules. Hence, the authors created a UV-absorbing chromophore by periodate oxidation of sphingosine following the saponification of the fatty-acyl amide bond of gangliosides or sphingolipids to produce a trans-α,β-unsaturated-alkene-aldehyde that will show a UV-absorption maxima at 220 nm. HPLC analysis of the trans-α,β-unsaturated-alkene-aldehyde was conducted using an internal reference method to improve assay accuracy. This was done by mixing a fixed amount of cis-α,β-unsaturated-alkene aldehyde[V] or cis-3-alkene-1,2-glycol[IV] with a sample extract. The internal reference compounds [IV] or [V] were synthesized in our laboratory starting from 1,2-5,6-mannitol- diacetonide[I] by a series of organic reactions including; 1) periodate oxidation of [I] to give the glyceraldehyde-acetonide[II], 2) Wittig synthesis to give the 3-alkene- 1,2-glycol-acetonide[III], 3) removal of acetonide protection by acid hydrolysis to give [IV], and 4) a second periodate oxidation to give [V] as shown in Scheme 1. The stereo-structure of the α,β-unsaturated-

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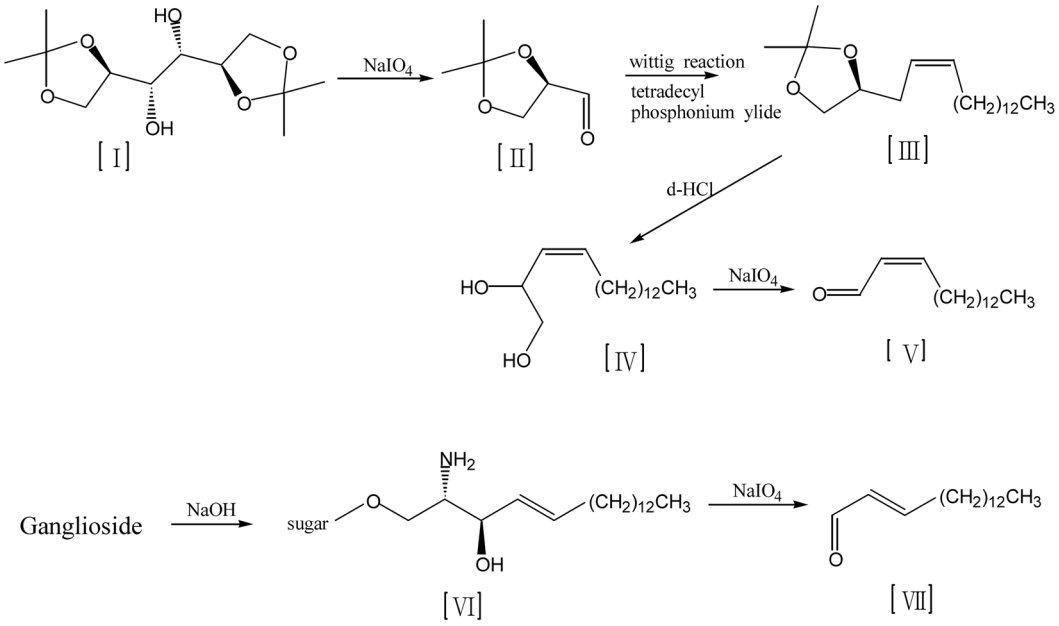

Scheme 1. [I]; 1,2,5,6-diisopropylidene mannitol, [II]; glycelaldehyde acetonide, [III]; cis-1,2-heptadecyl-3-ene-glycol-acetonide, [IV]; cis-3-heptadecene-1,2-diol [V]; cis-2-hexadecene aldehyde, [VI]; sphingosine glycoside, [VII]; trans-2-hexadecene aldehyde.

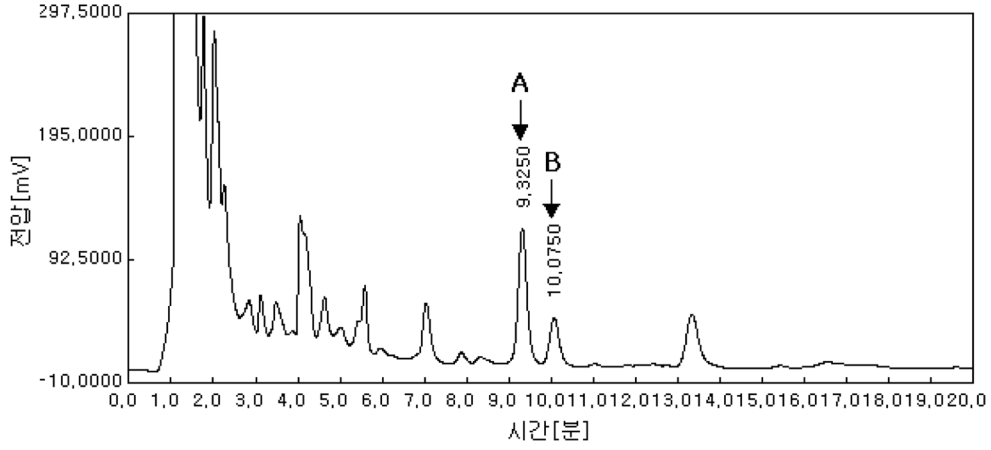

Fig. 1. HPLC analysis of water extracts of deer antlers.
A; Cis-2-hexadecene aldehyde (internal reference), B; trans-2-hexadecene aldehyde.

alkene-aldehyde produced by periodate oxidation of natural sphingosine was identified as the trans-isomer, and the chemically synthesized one as the cis-isomer by coupling constant analysis of olefinic protons on PMR-spectra of both compounds. As shown in Fig. 1, both stereo isomers having the same alkenyl chain length gave retention times on HPLC that allowed us to clearly resolve the isomer. Hence, the synthetically obtained cis-isomer can be used as a good internal reference for the analysis of natural sphingolipids.

This newly established HPLC-procedure was employed for the comparisons of sphingosine contents in place of gangliosides in the various parts of deer antlers ("tip-, upper-, mid- and base-part") as shown in Fig. 2, and also in the same medicinal parts of deer antlers produced from different countries such as New Zealand, China and Russia.

Materials and Methods

Materials – Deer antlers (from China, Russia and New Zealand) and ossified deer antlers (from Russia) were

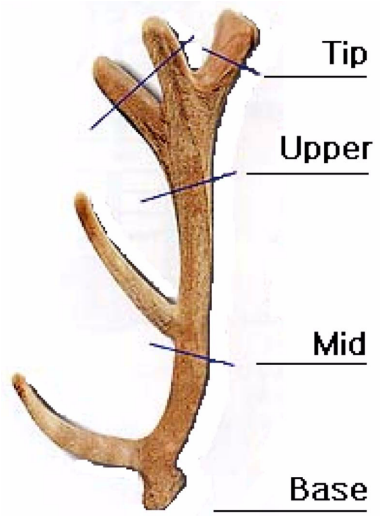

Fig. 2. Medicinal parts of deer antlers.

purchased from markets in Seoul, Korea. These were burned on an open flame to remove surface fur and shattered into small pieces. D-Sphingosine, 1,2: 5,6-Di-O-isopropylidene-d-mannitol and 2M BuLi in cyclohexane were purchased from Sigma-Aldrich and 1-tetradecyl-triphenyl-phosphonium bromide from Tokyo Kasei Co. (TCI).

Synthesis of cis-3-Heptadecene-1,2-diol (internal reference compound)[IV]

1) Synthesis of 1-tetradecyl-triphenyl-phosphonium-ylide – 5 mL of 2 M BuLi (in cyclohexane) was added to a suspension of tetradecyl-triphenyl-phosphonium bromide 5.0 g (9.3 mmol) in THF under nitrogen to produce an intense red solution of tetradecyl-triphenyl-phosphonium-ylide.

2) Synthesis of isopropylidene glyceraldehyde[II] – To 1.31 g (5 mmol) of 1,2:5,6- Di-O-isopropylidene-d-mannitol[I] dissolved in 10 mL of CH_2Cl_2, 500 μL of saturated $NaHCO_3$ solution and 2.1 g solid $NaIO_4$ were added and vortex-mixed vigorously for 2 h to oxidize [I] to produce isopropylidene glyceraldehyde[II]. The dichloromethane layer containing isopropylidene glyceraldehyde was separated and dehydrated by overnight stirring with 2.0 g anhydrous $MgSO_4$.

3) Synthesis of cis-1,2-heptadecyl-3-ene-glycol-acetonide[III] – To the THF solution of intense red tetradecyl-triphenyl-phosphonium-ylide under nitrogen, the dehydrated dichloromethane solution of [II] was added slowly under ice cooling until the intense red color had completely faded due to the Wittig reaction. The reaction mixture was added water and extracted with hexane to obtain [III]. The hexane layer was evaporated to give an amorphous solid reaction product [III] with some impurities. The amorphous residue was chromatographed on a silica-gel column to give a colorless oil of [III] (0.71 g, isolation yield; 24.6%) by elution with hexane/ethyl acetate (15 : 1). PMR data in $CDCl_3$ (400 MHz); δ_{TMS} 0.90 (3H, t, J = 7.2), 1.27~1.40 (22H, m, methylene-($C_{11}\underline{H}_{22}$), 1.41 and 1.44 (6H, s, isopropylidene two methyl), 2.07~2.14 (2H, m, olefin-adjacent -CH_2-), 3.52 (1H, dd, $C\underline{H}_aH_b$(-O-), J = 8.0), 4.07 (1H, dd, $CH_a\underline{H}_b$(-O-) J = 6.0, 8.0), 4.87 (1H, m, olefin adjacent-C$\underline{H}$(-O-)), 5.41 (1H, dd, J = 10.8, 8.4, isopropylidene adjacent olefin -CH=C$\underline{H}$CH(-O-)), 5.64 (1H, dt, J = 10.8, 7.2, alkyl-C$\underline{H}$=CH).

4) Deprotection of [III] to give cis-3-heptadecene-1,2-diol[IV] – [III] was dissolved in ethanol and stirred for 1 h under the addition of a few drop of d-HCl to deprotect the isopropylidene group. It was extracted with chloroform twice to give white crystalline powders of [IV] (0.59 g). PMR data in $CDCl_3$; δ_{TMS} 0.90 (3H, t, J = 6.8), 1.25~1.39 (22H, m, aliphatic methylenes), 1.95 (2H, s-like,-OH), 2.12 (2H, m, methylene protons adjacent to olefin), 3.49~3.62 (2H, m, C$\underline{H}_2$OH). 4.58 (1H, m, olefin adjacent C$\underline{H}$OH), 5.40 (1H, dd, J = 10.8, 8.4, -CH=C$\underline{H}$-CHOH-), 5.61 (1H, dt, J = 10.8, 7.2, alkyl-C$\underline{H}$=CH-).

5) Preparation of trans-2-hexadecene aldehyde[VII] from sphingosine and cis-2-hexadecene aldehyde[V] from [IV] – Sphingosine (100 mg) dissolved in 5 mL CH_2Cl_2 was oxidized by vortex-mixing for 10 min with 1 g of $NaIO_4$ solution in water. Dichloromethane layer gave a UV-absorbing single spot of Rf. 0.51 of trans-2-hexadecene aldehyde on TLC (developing solvent; Hexane/Ethyl acetate (5 : 1)). PMR data in $CDCl_3$; δ_{TMS} 0.89 (3H, t, J = 7.0 methyl), 1.19~1.45 (22H, m, alkyl), 2.35 (2H, m, olefin-adjacent methylene), 6.12 (1H, dd, J = 8.0, 15.5, RCH=C$\underline{H}$CHO), 6.86 (1H, dt, J = 6.5, 15.5, RC$\underline{H}$=CHCHO), 9.52 (1H, d, J = 8.0, R-C$\underline{H}$O).

[IV] was also oxidized by the same procedure to give cis-2-hexadecene aldehyde on Rf. 0.49. PMR data in $CDCl_3$; δ_{TMS} 0.89 (3H, t, J = 7.0, methyl), 1.19~1.43 (22H, m, alkyl), 2.62 (2H, m, RC$\underline{H}_2$CH=CH), 5.97 (1H, dd, J = 9.5, 11.0, RCH=C$\underline{H}$CHO), 6.65 (1H, dt, J = 8.5, 11.0, RC$\underline{H}$=CHCHO), 10.09 (1H, d, J = 8.0, aldehyde). Dichloromethane was removed from both reaction products by warming in an 85 °C water bath to give an amorphous waxy white solid ([VII]) and a colorless oil ([V]). Both the trans- and cis-2-hexadecene aldehyde demonstrated the same UV-absorption-spectra giving a λ_{max} at 220 nm in methanol. [IV] or [V] may be used as

Table 1. Comparison of sphingosine contents in deer antlers of various medicinal parts and also based on producing countries (extracted by method-2)

	Tip	Upper	Mid	Base
New Zealand	46.22 pmol/g	46.08 pmol/g	41.99 pmol/g	–
Russia	44.69 pmol/g	42.28 pmol/g	41.13 pmol/g	34.81 pmol/g
China	43.80 pmol/g	40.61 pmol/g	38.74 pmol/g	–

an internal reference compound in the HPLC-analysis of [VII] which will be derived quantitatively from gangliosides or other sphingo-lipids contained in natural products.

HPLC-analysis – Sphingosines found in nature have trans-olefinic configuration (Carter *et al*., 1947) hence all sphingo-lipids in nature will give only trans-αβ-unsaturated alkyl-aldehydes when the sphingo-lipids are treated by periodate-oxidation after saponification by alkali treatment. On the other hand, the authors developed a total synthesis procedure for [V] from [I] through four step organic reactions as shown in Scheme 1.

[V] is suitable as the internal reference compound in the HPLC-analysis of [VII] which will be produced from various sphingo-lipids through two step chemical reactions, i.e. 1) saponification and 2) periodate oxidation. Trans- and cis-2-hexadecene aldehyde, which were synthesized in our laboratory, were chromatographed using a Waters™ 600 pump with a Waters™ 486 UV detector on a reversed phase silica column (Mightsil RP-18, 150 × 4.6 mm, 5 μm) using a mixed solvent of acetonitrile/water (90 : 10, v/v) as the mobile phase (flow rate; 1.5 mL/min) and monitored by UV-absorption at 220 nm to give a clearly resolved trans isomer peak at Rt. 10.075 min and a cis-isomer peak at Rt. 9.325 min without any peak overlap.

Calibration curve preparation – Stock-solution-A as internal reference (0.2 mM-cis-3-heptadecene-1,2-diol in CH_2Cl_2) and stock-solution-B (1 mM-sphingosine in CH_2Cl_2) were prepared. Calibration samples were prepared by mixing fixed amounts (500 μL) of stock solution A to each tube, differential amounts of stock solution-B (100 μL, 200 μL, 300 μL, or 400 μL) and differential amounts of CH_2Cl_2 to adjust the final volume to 1 mL. To these calibration samples, 100 μL of 10%-$NaIO_4$ solutions were added and vortex-mixed for 10 min to oxidize the sample and internal reference concomitantly. After centrifugation, 20 μL of lower phases were injected for HPLC-analysis. The calibration curve obtained by HPLC showed almost strict linearity in the range of 1 - 4 pmol in one experiment giving a correlation coefficient of R = 0.9995 (data not shown). Considering the avoidance of peak-overlap and scale out, the concentration range will be much more expanded to 400 - 500 pmol without the loss of linearity.

Comparisons of sphingosine contents of different medicinal parts of deer antlers and deer antlers having different countries of origin – To compare the sphingosine contents of various antler samples, 5 g of analytical samples were collected from four different parts of antler (i.e. tip-, upper-, mid- and base-) (based on medicinal uses) of various deer antlers originating from New Zealand, Russia and China. The samples were extracted by Method-2 and treated by the above mentioned processes appearing in the 'calibration curve preparation' to analyze the contents of α,β-unsaturated aldehyde ([VII]) in place of gangliosides by using [IV] or [V] as internal reference on reversed-phase HPLC. The results are tabulated in Table 1.

Sphingosine contents of deer antler and ossified deer antler

Extraction Method-1 – Five g portions of deer antler and ossified deer antler were extracted exhaustively by refluxing with 50 mL of a mixed solvent of chloroform/methanol (1 : 1). The extracts were vacuum distilled to dryness. The glycol-lipid fractions of the residues (ganglioside rich fractions) were separated by the Folch-Suzuki method (Folch *et al*., 1957) partitioning the material in chloroform/methanol/0.88% KCl (8 : 4 : 3). The upper phase was separated and vacuum distilled to give a glycolipid fraction containing gangliosides as major spots.

Extraction Method-2 – Five g portions of deer antler and ossified deer antler were extracted exhaustively by heating 3 h with 5-volumes of distilled water at 135 in an autoclave. The aqueous extracts were lyophilized to give a sticky solid mass.

Comparisons of sphingosine contents in deer antler and ossified deer antler –

Sphingosine contents of the extracts of deer antler and ossified deer antler were analyzed by the internal reference method of the HPLC using a reversed phase column and the pre-treatment method in the "Calibration curve preparation" section above. The results were tabulated (Table 2).

Table 2. Total sphingosine contents in deer antlers and ossified deer antlers

Sample	pmol / 1 g or (1 cm^3) of samples	
	Extraction method-1	Extraction method-2
Deer antler	42.37 pmol/g (33.47 pmol/cm^3)	34.95 pmol/g (27.61 pmol/cm^3)
Ossified deer antler	3.01 pmol/g (3.58 pmol/cm^3)	14.25 pmol/g (16.96 pmol/cm^3)

Table 3. Total sphingosine contents in commercial collagens

Collagens	pmol / 1 g of collagen
Deer antler collagen	118.83
Ossified deer antler collagen	34.72
Fish collagen	not detected
Porcine collagen	not detected

Analysis of sphingosine impurities in technical grade porcine collagen and fish collagen – 1 g samples of technical grade porcine collagen, fish collagen and collagen-rich extracts of deer antler and ossified deer antler were treated by the methods described above to analyze their sphingosine content. The results are shown in Table 3.

Results and Discussion

Development of a new sensitive HPLC-assay for sphingosine – Gangliosides are distributed in brain and neural cells (Schnaar *et al*, 2009), and have various important biological functions such as memory storage (Fong *et al*., 1997). Hence, developing a new sensitive micro-assay procedure for sphingolipids by HPLC will be useful in the field of biomedical sciences.

The present paper is concerned with the development of an HPLC assay procedure for sphingo-lipids by creating a UV-absorbing chromophore on the sphingosine moiety of sphingolipids. Gangliosides are sphingo-lipids having fatty acyl amides of sphingosine triosides including sialic acid as a part of the glycosyl moiety. When gangliosides are saponified, sphingosine triosides will be produced. These substances have a vicinal amino-hydroxyl group on the sphingosine moiety. This vicinal amino-hydroxyl group on the sphingosine skeleton is an easy target of periodate oxidation and produces trans-α,β-unsaturated-alkene aldehyde, which has a UV-absorption maxima around 220 nm.

This UV-absorbing chromophore will enable the quantitative analysis of sphingosines or gangliosides by HPLC. To improve assay accuracy, the internal reference procedure was adopted.

Synthesis of internal reference compounds cis-3-Heptadecene-1,2-diol[IV] and cis-2-hexadecene-aldehyde [V] – To improve assay accuracy, [IV] or [V] were adopted as internal reference compounds, which were synthesized in our laboratory starting from [I] through three or four step organic reactions. These two compounds were synthesized with good purity for use as the internal reference compounds in the HPLC-assay of [VII] derived from sphingo-lipids. [VII] and [V] showed good resolution in the retention times of HPLC without any peak overlap. [IV] is preferred over [V] to mix with sample extracts before periodate oxidation due to the higher chemical stability of [IV].

Some examples of HPLC-application in the analysis of deer antler extracts – The results of sphingosine analysis in various medicinal parts of deer antlers showed highly matching results to the traditional reputation on the efficacy order of tip-, > upper-, > mid- and > base-part as shown in Table 1.

However the sphingosine contents in the same medicinal parts of deer antlers from different antler producing countries showed small differences with decreasing order of sphingosine contents with decreasing order of New Zealand > Russia > China as shown in Table 1.

Comparison of sphingosine contents of extracts of deer antler and ossified deer antler – The present assay procedure was also used to compare the sphingosine contents of ordinary deer antler and ossified deer antler.

When the samples of deer antler and ossified deer antler were extracted by reflux in organic solvents (chloroform/methanol system), the sphingosine contents of ossified deer antler showed a much reduced content than that of un-ossified deer antler, possibly due to insufficient extraction of ossified deer antler. However, when the samples were extracted in 135 ºC-water in the autoclave, the sphingosine contents of ossified deer antler were much higher than that of the organic solvent extraction (Table 2). These data suggest that bioactive components of ossified deer antler may not show big differences compared to those of deer antler when water extractions are conducted at elevated temperature such as 130 - 135 ºC.

The major chemical components of the extracts of deer antler and ossified deer antler are gelatins which were produced by heat induced denaturation of type-1 and type-2 collagens contained in deer antler and ossified deer antler during the extraction process. When we consider future markets for some commercial products produced from the extracts of deer antler or ossified deer antler as a major component, the possibility of the appearance of some illegal, adultered products produced by mixing commercial grade fish collagen or porcine collagen, or their gelatins obtained by heat treatment, in place of heat treatment of deer antler or ossified deer antler, should be considered. Hence various collagen or gelatin containing products were analyzed for sphingosine contents by this newly established assay procedure as shown in Table 3.

As shown in Table 3. the water extracts of deer antler and ossified deer antler have high concentrations of sphingosine. However, porcine collagen and fish collagen contain no sphingosine at all. These data will contribute to future marketing and to quality inspections of the commercial products of deer antler or ossified deer antler to identify adultered products produced by mixing some commercial "porcine collagen" or "fish collagen" in place of the extracts of deer antler or ossified deer antler.

Conclusion

This paper describes a new HPLC-assay procedure for sphingosine by creating a UV-absorbing chromophore trans-2-hexadecene aldehyde from the sphingosine moiety of various sphingo-lipids. This assay procedure is suitable for the analysis of sphingo-lipids. However, the ganglioside content in deer antler may be assayed by this method when the ganglioside fraction is appropriately obtained by a partial purification process.

This HPLC-assay procedure was used to extract gangliosides from deer antler and ossified deer antler. The ganglioside content in ossified deer antler extracted by reflux in organic solvents shows much less end product than deer antler due to poor extraction. However, when the ossified deer antler was extracted by heating in 135 ºC water, the ganglioside content was highly elevated (Table 2).

Any adultered products of deer antler or ossified deer antler produced by mixing commercial grade collagens as fish collagen or porcine collagen will be able to be detected by analysis of the sphingosine content.

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