

Technical paper

Determination of Long-chain Alcohol and Aldehyde Contents in the Non-Centrifuged Cane Sugar Kokuto

Yonathan ASIKIN^{1,2}, Takeshi CHINEN¹, Kensaku TAKARA¹ and Koji WADA^{1*}

¹ Department of Bioscience and Biotechnology, Faculty of Agriculture, University of the Ryukyus, Senbaru-1, Nishihara-cho, Okinawa 903-0213, Japan

² Department of Food Science and Technology, Faculty of Agricultural Technology, Bogor Agricultural University, Kampus IPB Darmaga, Bogor 16002, Indonesia

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The long-chain alcohol and aldehyde contents and compositions were determined in seven types of the non-centrifuged cane sugar kokuto (Kokuto A to G). Long-chain alcohols, known as policosanols, have been reported to have beneficial effects on human health. Policosanols were extracted effectively with hexane/methanol (20:1 v/v) and long-chain aldehydes were extracted with chloroform/methanol (2:1 v/v). These compounds were then analyzed by gas chromatography and gas chromatography-mass spectrometry. Trimethylsilyl ethers, the policosanols fragments, and a number of unique aldehyde fragments were analyzed to identify the source compounds. Octacosanol (C28-OH) was confirmed to be the main component in all kokuto samples. Moreover, the production process influenced the policosanols and long-chain aldehyde contents in kokuto. Kokuto A, which was produced by an open pan boiling method, showed the highest content of policosanols (86 mg/100g) and long-chain aldehydes (9 mg/100 g).

Keywords: policosanols, aldehyde, cane sugar, kokuto

Introduction

Kokuto, a unique brown cane sugar, has been traditionally produced without molasses removal by a non-centrifugal method in Okinawa, Japan. In several previous studies, the presence of antioxidants and phenolic compounds in kokuto has been investigated (Takara *et al.*, 2002, 2003). In this study, we focused on policosanols and long-chain aldehydes. Policosanols are a group of long-chain (C20 - C30) aliphatic primary alcohols that are of great interest due to their beneficial effects on human health, for example, reducing platelet aggregation, reducing low-density lipoprotein levels in blood, and inhibiting cholesterol synthesis and ergogenic properties (Castano *et al.*, 2003; Singh *et al.*, 2006; Taylor *et al.*, 2003). Aldehydes are some of the main components of natural wax extracted from plants (Adhikari *et al.*, 2006) and straight-chain aldehydes are some of the lipid biomarkers seen in plant leaves and roots (Jansen *et al.*, 2006).

Recently, research in policosanols analysis using various materials and techniques has been widely reported (Adhikari *et al.*, 2006; Wang *et al.*, 2007; Wu *et al.*, 2007). Sugarcane and its wax have been reported to contain numerous policosanols and are used as major sources for commercial extraction (Irmak *et al.*, 2006; Marrison *et al.*, 2006; Nuissier *et al.*, 2002). However, there is little information on long-chain aldehydes, particularly in sugarcane and its products. Thus, the present study investigated the content of both policosanols and long-chain aldehydes in several samples of the non-centrifuged cane sugar kokuto.

Materials and Methods

Samples Seven types of kokuto (Kokuto A to G; produced on Aguni, Hateruma, Iheya, Iriomote, Kohama, Tarama and Yonaguni islands of Okinawa, Japan) from the 2007 - 2008 production year were obtained from each kokuto manufacturer and used as source materials. Cane juice and samples from the kokuto production line were also used in this study.

*To whom correspondence should be addressed.

Email: kojiwada@agr.u-ryukyu.ac.jp

Materials and Reagents The policosanols standards consisted of docosanol (C22), tetracosanol (C24), hexacosanol (C26), octacosanol (C28) and triacontanol (C30), which were purchased from Sigma (Sigma Chemical, St. Louis, MO, USA). The derivatization reagent *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) was purchased from GL Science (Tokyo, Japan). Pyridinium chlorochromate (Sigma Chemical) was used in the synthesis of long-chain aldehyde standards. All other reagents were of analytical grade unless otherwise specified.

Standard Preparation A mixture of the policosanols standards was prepared in toluene for policosanols compound quantification. The policosanols standards were also prepared in chloroform and derivatized with MSTFA (2:1 v/v) at 50°C for 15 min for mass spectrum identification. The aldehyde standards were synthesized from their alcohol forms by oxidation with pyridinium chlorochromate as described by Pérez-Camino *et al.* (2003). The corresponding 1 mM alcohol standards (19.14 mg hexacosanol, 20.54 mg octacosanol, and 21.94 mg triacontanol) and 9 mM pyridinium chlorochromate (97.5 mg) were stirred in 50 ml of dichloromethane for 1.5 h at room temperature. The reaction mixture was eluted with dichloromethane through a short column (6 × 2 cm i.d.) packed with silica gel-60. The reaction products were then dried with N₂ and diluted in toluene. The synthesized long-chain aldehyde standards were then subjected to gas chromatography (GC) analysis.

Sample Extraction Kokuto samples were crushed and ground with a dry blender before use. Samples were extracted by two methods: the liquid-liquid extraction (LLE) method according to Irmak *et al.* (2006) and the solid-liquid extraction (Soxhlet) method. All dry residue extracts were diluted with toluene for GC analysis or with chloroform for GC-mass spectrometry (MS) analysis. All extractions and analyses were conducted in triplicate.

LLE Method Briefly, 6 g of kokuto sample was mixed with 50 ml of 1.0 M NaOH in 50 ml of methanol and subsequently hydrolyzed by reflux for 30 min. After cooling, the mixture was filtered through Advantec No. 5A filter paper under vacuum. Pure water was then added to the filtrate. The solution was extracted three times with diethyl ether. The combined diethyl ether phases from the three extractions were washed with pure water until the water phase was pH 7. The extract was dried over 50 g of anhydrous sodium sulfate by storing at 4°C overnight. The solvent was then removed from the extract with a rotary evaporator under vacuum. The dry extract was diluted with toluene or chloroform to obtain a 2-ml sample volume for analysis.

Soxhlet Method Briefly, 6 g of kokuto or 20 g of freeze-dried cane juice were placed in an Advantec No. 84 thimble

filter and extracted using the Soxhlet method with several systems of organic solvents of about 150 ml each. The solvent systems included Soxhlet A (chloroform/methanol, 2:1 v/v); Soxhlet B (hexane/methanol, 10:1 v/v); Soxhlet C (hexane/methanol, 20:1 v/v); Soxhlet D (hexane/methanol, 30:1 v/v); and Soxhlet E (hexane). Solvent solutions were removed from the extract using a rotary evaporator under vacuum at 40°C. Dry extracts were then diluted with toluene or chloroform to obtain 2-ml sample volumes for analysis. The extraction time was also varied to investigate the heat stability and optimal extraction conditions of policosanols and long-chain aldehydes.

GC Analysis A Shimadzu GC 17-A equipped with a fused capillary column (DB 5, 0.25 mm i.d. × 30 m; J&W Scientific, Folsom, CA, USA) and a flame ionization detector were used for quantitative analysis of policosanols and long-chain aldehydes. The GC injector and the flame ionized detector were both set at 350°C. Samples (1 µl) were injected with a split ratio of 1:10 under helium atmosphere. The oven temperature was initially set to 150°C, increased to 320°C at 4°C/min, and then maintained at 320°C for 15 min. The relationship between concentration and peak height was calibrated by injecting mixture standards of policosanols and aldehydes of different concentrations over the concentration levels of the extract samples.

GC-MS Analysis The mass spectra of policosanols were analyzed by silylated derivatization; however, the mass spectra of aldehydes were analyzed without silylation. Policosanols were identified by their trimethylsilyl derivatives. MSTFA was used as the silylation reagent. The mixed derivatization solution, which included 0.5 ml of sample in chloroform and 250 µl of MSTFA, was heated at 50°C for 15 min, followed by the addition of chloroform to obtain a 1-ml sample for analysis. Analysis was performed with a Shimadzu GC-MS QP-2010 equipped with a fused capillary column DB-5 MS (0.25 mm i.d. × 30 m, J&W Scientific) under the same GC conditions described above. Samples (0.3 µl) were injected with a split ratio of 1:10. For MS detection, the electron impact (EI) ion source and transfer line temperatures were set to 200 and 280°C, respectively, and the ionization energy was set to 70 eV. The mass acquisition scan range and rate were 30 - 500 amu and 2 scans/s, respectively.

Results and Discussion

Figure 1 shows the typical chromatograms of both standards and kokuto sample extracts. All compounds in the standard mixture were completely separated to single peaks (Fig. 1A). The retention times of the three synthesized aldehyde compounds, hexacosanal, octacosanal and triacontanol, were 1 min less than their corresponding alcohol compounds.

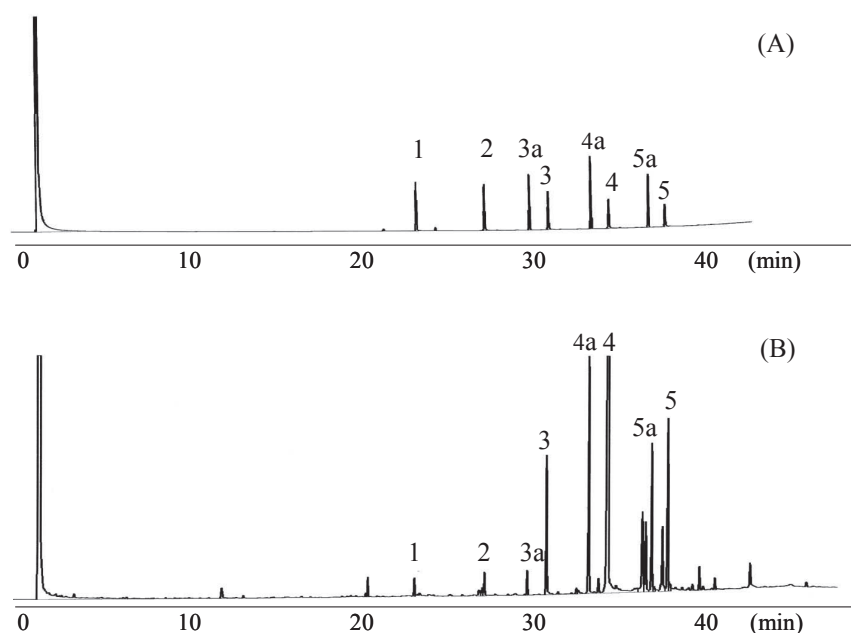


Fig. 1. Typical gas chromatograms of policosanol and long-chain aldehyde standards (A) and Kokuto A (B). A fused capillary column (DB 5, 0.25 mm i.d. $\times$ 30 m) and a flame ionization detector were used. Both GC injector and flame ionized detector were set at 350°C. Samples (1 μ l) were injected with a split ratio of 1:10. The oven temperature was initially set to 150°C, increased to 320°C at 4°C/min, and then maintained at 320°C for 15 min. Peak 1: docosanol; Peak 2: tetracosanol; Peak 3: hexacosanol; Peak 3a: hexacosanal; Peak 4: octacosanol; Peak 4a: octacosanal; Peak 5: triacontanol; Peak 5a: triacontanal.

Quantification of each policosanol and aldehyde compound in the extracted samples was determined based on the retention time and peak height of each standard. Although MSTFA has been reportedly used in policosanol determination (Adhikari *et al.*, 2006; Marrison *et al.*, 2006), we found that MSTFA was less effective for policosanol determination when compared to direct analysis. The use of an internal standard (i.e., octacosanoic acid) was also avoided due to a number of closed peaks in areas of interest on the sample chromatograms (Fig. 1B).

Policosanols were identified based on the mass fragment patterns of EI spectra of the trimethylsilyl derivatives. For example, the mass fragment of m/z 467 was a specific target ion of the trimethylsilyl ether C28 and the qualifier ions were m/z 103, 468 and 469. In the aldehyde group, the mass fragment pattern of $C_{28}H_{56}O$, though weak, was clearly recognizable from the mass fragments of m/z 362 (M-46, loss of $CH_2=CH_2$ and H_2O from $C_{28}H_{56}O^+$), m/z 364 (M-44, loss of ion $CH_2=CH-O^+$ from $C_{28}H_{56}O^+$), m/z 390 (M-18, loss of H_2O from $C_{28}H_{56}O^+$), and m/z 408 (M^+ , $C_{28}H_{56}O^+$ ion), which are characteristic fragments of aldehydes. These policosanol and aldehyde fragmentation patterns were similar to previous reports (Irmak *et al.*, 2006; Pérez-Camino *et al.*, 2003).

The LLE and Soxhlet methods using several solvent systems were compared to investigate their effectiveness

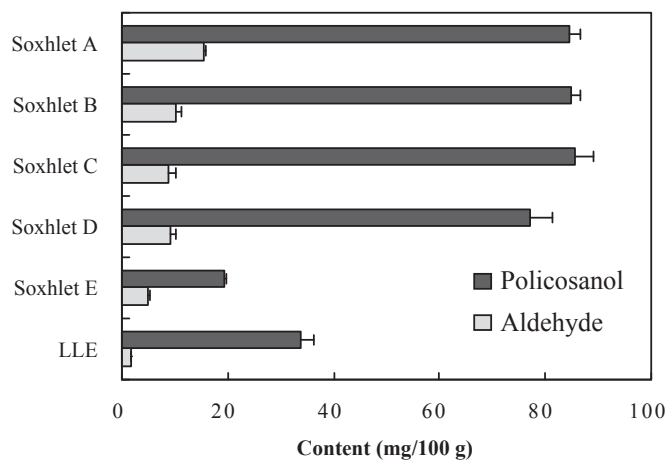


Fig. 2. Comparison of methods and solvent types on policosanol and long-chain aldehyde extraction of Kokuto A. Samples were extracted for 24 h by two methods, the liquid-liquid extraction (LLE) method and the solid-liquid extraction (Soxhlet) method. Soxhlet A: chloroform/methanol (2:1 v/v); Soxhlet B: hexane/methanol (10:1 v/v); Soxhlet C: hexane/methanol (20:1 v/v); Soxhlet D: hexane/methanol (30:1 v/v); Soxhlet E: hexane. Values are expressed as mean $\pm$ standard deviation ($n = 3$).

for policosanol and long-chain aldehyde compounds (Fig. 2). Among the systems, Soxhlet A, B and C were effective for extraction of policosanol compounds. Soxhlet C, which used hexane/methanol (20:1 v/v), showed the highest yield.

Soxhlet A, which used chloroform/methanol (2:1 v/v), was slightly better at extracting aldehyde compounds compared with other methods. Hexane is generally used for extracting wax components, but we found that extraction with methanol is more effective; as methanol is miscible with water and increases the penetrating strength of the solvent through matrix samples.

Figure 3 shows the effects of extraction time on policosanols and long-chain aldehyde extraction from Kokuto A. The policosanols yield was optimal at 24 h of extraction with hexane/methanol (20:1 v/v) and was unaffected by further heating. Small amounts of aldehyde compounds in Kokuto A were decomposed by heating. An excellent yield of long-chain aldehydes was obtained at 8 h of extraction with hexane/methanol (20:1 v/v), and further heating led to oxidation and slightly decreased the aldehyde content.

To simultaneously determine the policosanols and long-chain aldehyde contents of kokuto samples, the Soxhlet method using hexane/methanol (20:1 v/v) for 24 h extraction was used. Tables 1 and 2 respectively show the policosanols and long-chain aldehyde contents of the seven types of kokuto. The highest total policosanols content was seen in Kokuto A (86 mg/100 g). The GC analysis of policosanols determined that Kokuto A was comprised of 0.5% docosanol (C22-OH), 0.8% tetracosanol (C24-OH), 10.7% hexacosanol (C26-OH), 77.0% octacosanol (C28-OH) and 10.9% triacontanol (C30-OH), which confirms previous studies on sugarcane policosanols that octacosanol was the main component in kokuto (Irmak *et al.*, 2006; Marrison *et al.*, 2006). Policosanols were found at somewhat lower amounts in other kokuto samples than in Kokuto A. Kokuto B contained about 12 mg/100 g policosanols with 69% being C28, while

Kokuto C to G contained 7 - 9 mg/100 g policosanols, of which about 54 - 59% was C28 and none was C24. However, the content of long-chain aldehydes was lower than that of policosanols in all kokuto samples (Table 2). The highest long-chain aldehyde content was seen in Kokuto A with only 9 mg/100 g aldehydes. The GC analysis of aldehydes determined that Kokuto A was comprised of 12.7% hexacosanal (C26-CHO), 67.7% octacosanal (C28-CHO) and 19.6% triacontanal (C30-CHO). Kokuto B contained only 1 mg octacosanal/100 g aldehydes. On the other hand, long-chain aldehydes (C26-CHO, C28-CHO and C30-CHO) were not detected in Kokuto C to G. We suggest that these unique results are due to differences in the production process (Fig. 4).

In the production process of kokuto, sugarcane juice is filtered, concentrated without molasses removal, and is then crystallized. In this process, kokuto contains several bioac-

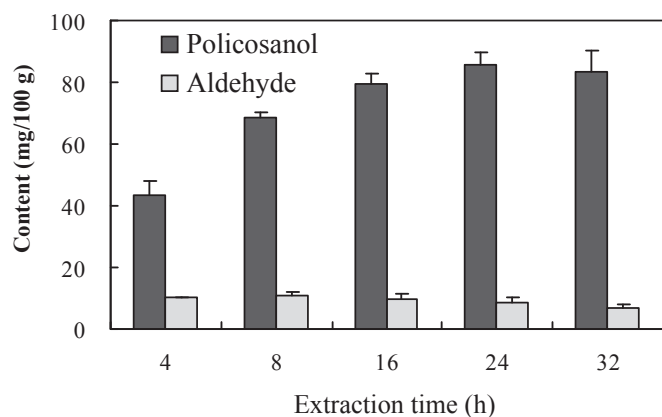


Fig. 3. Comparison of the solid-liquid extraction time of policosanols and long-chain aldehyde extraction from Kokuto A. Policosanols and long-chain aldehydes from Kokuto A were extracted with hexane/methanol (20:1 v/v) by the solid-liquid extraction method. Values are expressed as mean $\pm$ standard deviation ($n = 3$).

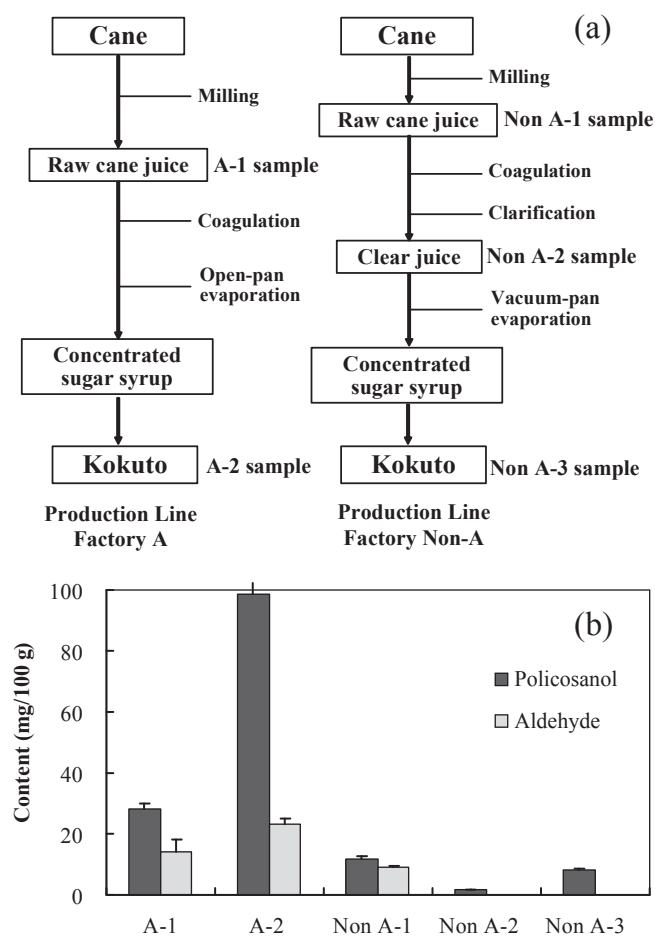


Fig. 4. Production processes of kokuto (a) and the policosanols and long-chain aldehyde contents in kokuto samples from different production lines (b). Policosanols and long-chain aldehydes from samples were extracted with hexane/methanol (20:1 v/v) for 24 h by the solid-liquid extraction method. A-1: cane juice from production line A; A-2: Kokuto A; Non A-1: cane juice from production line Non A; Non A-2: filtered cane juice from production line Non A; Non A-3: Kokuto Non A. Values are expressed as mean $\pm$ standard deviation ($n = 3$).

Table 1. Policosanol content of the seven types of kokuto.

Kokuto ^a	Policosanol content (mg/100 g) ^b					Total
	C22-OH	C24-OH	C26-OH	C28-OH	C30-OH	
A	0.46 ± 0.06	0.68 ± 0.57	9.18 ± 0.62	65.99 ± 2.03	9.37 ± 1.12	85.69 ± 3.45
B	0.48 ± 0.06	0.16 ± 0.02	0.94 ± 0.07	8.63 ± 0.77	2.28 ± 1.11	12.48 ± 0.72
C	0.49 ± 0.06	n.d. ^c	0.43 ± 0.04	5.07 ± 0.52	2.56 ± 0.28	8.64 ± 0.77
D	0.44 ± 0.09	n.d.	0.32 ± 0.06	3.82 ± 0.60	2.44 ± 0.32	7.02 ± 0.97
E	0.41 ± 0.03	n.d.	0.35 ± 0.06	4.27 ± 0.68	2.27 ± 0.29	7.29 ± 1.04
F	0.40 ± 0.01	n.d.	0.33 ± 0.04	4.00 ± 0.08	2.23 ± 0.21	6.96 ± 0.23
G	0.44 ± 0.05	n.d.	0.40 ± 0.04	4.77 ± 0.31	2.52 ± 0.16	8.13 ± 0.44

^a Samples were extracted with hexane/methanol (20:1 v/v) by the solid-liquid extraction method for 24 h.

^b Values are expressed as means ± standard deviation (n = 3).

^c Not detected.

Table 2. Long-chain aldehyde content of the seven types of kokuto.

Kokuto ^a	Long-chain aldehyde content (mg/100 g) ^b			Total
	C26-CHO	C28-CHO	C30-CHO	
A	1.10 ± 0.03	5.89 ± 1.42	1.70 ± 0.11	8.70 ± 1.56
B	n.d. ^c	1.05 ± 0.05	n.d.	1.05 ± 0.05
C	n.d.	n.d.	n.d.	n.d.
D	n.d.	n.d.	n.d.	n.d.
E	n.d.	n.d.	n.d.	n.d.
F	n.d.	n.d.	n.d.	n.d.
G	n.d.	n.d.	n.d.	n.d.

^a Samples were extracted with hexane/methanol (20:1 v/v) by the solid-liquid extraction method for 24 h.

^b Values are expressed as means ± standard deviation (n = 3).

^c Not detected.

tive compounds from sugarcane, including policosanols. There are two main production processes for Okinawan kokuto (Fig. 4a). Cane juice from both these production lines showed different concentrations of policosanols and long-chain aldehydes (Fig 4b). Kokuto A is traditionally manufactured with an open pan boiling technique in a series of pans located above a furnace. In this process, cane wax containing policosanols and aldehydes is expected to remain in the final product. Not surprisingly, Kokuto A contained policosanols at levels 8-fold higher than in other kokuto products (Fig 4b). However, the long-chain aldehyde content was found to be lower than the policosanols content in Kokuto A due to the high temperature (>100°C) that decomposes aldehyde compounds during production of Kokuto A. On the other hand, medium-scale sugars are processed with a modern vacuum pan technology for production of Kokuto B to G (Non A). The clarification process relies on a filter cake system in these production lines. Thus, wax components are removed with the filtered cake waste from the processed cane juice. As a result, the filtered cane juice (Non A-2) and final product (Non A-3) contain small amounts of wax components.

In conclusion, we determined the policosanols and long-chain aldehyde contents and clarified the differences in content of potent functional compounds in several types of kokuto.

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