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Characterization of Pine Nuts in the U.S. Market, Including Those Associated with "Pine Mouth", by GC-FID

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ABSTRACT: Taste disturbances following consumption of pine nuts, referred to as "pine mouth", have been reported by consumers in the United States and Europe. Nuts of *Pinus armandii* have been associated with pine mouth, and a diagnostic index (DI) measuring the content of Δ5-unsaturated fatty acids relative to that of their fatty acid precursors has been proposed for identifying nuts from this species. A 100 m SLB-IL 111 GC column was used to improve fatty acid separations, and 45 pine nut samples were analyzed, including pine mouth-associated samples. This study examined the use of a DI for the identification of mixtures of pine nut species and showed the limitation of morphological characteristics for species identification. DI values for many commercial samples did not match those of known reference species, indicating that the majority of pine nuts collected in the U.S. market, including those associated with pine mouth, are mixtures of nuts from different *Pinus* species.

KEYWORDS: *pine nuts, "pine mouth", diagnostic index, DI, SLB-IL 111 column, Δ5-unsaturated polymethylene-interrupted fatty acids*

INTRODUCTION

Nuts from certain *Pinus* species are highly nutritious foods because of their relatively high fat (ca. 40−60%) and protein (ca. 30−40%) contents.[1] Pine nuts have been a staple in the diets of several Native American tribes and for peoples across the Mediterranean region and Asia for hundreds or thousands of years.[2,3] Pine nuts are considered to be an abundant natural resource, and world production was estimated to be about 20000 tons/year.[4] Various species of pine nuts are eaten raw or roasted and are used for products such as pastry, sauces, and chocolates.

Nuts from *Pinus koraiensis*, *Pinus pinea* L., and *Pinus gerardiana* have been a part of global commerce for many years,[3] and *Pinus sibirica* has recently entered the international marketplace.[2] Nuts from the edible species *Pinus edulis*, *Pinus lambertiana*, and *Pinus monophylla* are primarily consumed in the United States. Until recently, nuts from *Pinus armandii*, primarily produced and consumed in Asia, and *Pinus cembra*, primarily consumed in Europe, were considered to be locally important but not major contributors to international trade compared with *P. koraiensis*, *P. pinea* L., and *P. gerardiana*.[2]

Some consumers have reported taste disturbances, commonly referred to as "pine mouth", following consumption of pine nuts. The first case of "pine mouth" was reported in Belgium in 2000.[5] Since then, several publications have reported consumer complaints of a bitter taste in the mouth, which can persist from 8 days to 2 weeks after the consumption of pine nuts.[6,7] The French Food Safety Agency[8] described a growing number of complaints of pine mouth syndrome among French consumers but was unable to identify a cause for the taste disturbances. In the largest overview to date, Flesch et al.[9] provided a descriptive report of more than 3000 cases of nut-related dysgeusia that were reported in France between May 2008 and January 2010. No species information was provided, and an etiological agent was not identified. Between July 2008 and November 2010, the U.S. Food and Drug Administration (FDA) received 197 consumer complaints of taste disturbances related to the consumption of pine nuts, and 15 samples associated with these complaints were collected and analyzed.[10] To date, no chemical compound in the collected samples has been identified as a potentially causative agent for the taste disturbances.

The French Food Safety Agency reported the presence of nuts from *P. armandii* in some of the products associated with consumer complaints.[8] *P. armandii* is not among the 29 species of pine nuts traditionally used for human consumption.[3] The identification of the species of pine nuts in commercial samples became an area of active investigation on the basis of the report by the French Food Safety Agency,[8] and the hypothesis was that consumption of pine nuts from *P. armandii*, a species not previously consumed in some markets, might be linked to the appearance of taste disturbances.[7]

Wolff et al.[11] demonstrated that the fatty acid (FA) composition of gymnosperms provided useful chemometric data for taxonomy and phylogeny of this group. Whereas the most abundant fatty acids (FAs) in conifer nuts are oleic acid (9-18:1) and linoleic acid (9,12-18:2), the Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA) are characteristic of *Pinus* species.[12,13] The primary Δ5-UPIFA in conifer nuts are taxoleic acid (5,9-18:2), pinolenic acid (5,9,12-18:2), and sciadonic acid (5,11,14-20:3).[14−16] All of the unsaturated FAs reported in pine nuts have double bonds in the *cis* configuration. Destaillats et al.[7] proposed the use of a diagnostic index (DI) calculated as the ratio between the Δ5-UPIFA and their metabolic FA precursors as a tool for identifying the botanical origin of pine nuts in commercial samples.

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In this study, we report the FA composition of a series of reference pine nuts and market samples, including pine mouth-associated samples. We evaluated the use of the DI for identification of pine nut species and compared the results of species identification obtained with the DI method with the results from a DNA-based method recently developed in our laboratory by Handy et al.[17]

MATERIALS AND METHODS

Collection of Samples. Shelled and unshelled pine nuts were purchased from a wide range of sources to obtain a variety of reference pine nuts and samples representative of the U.S. market. We obtained a total of 15 reference samples, 13 market samples, and 17 samples that were associated with consumer complaints of pine mouth.

Reference samples contained nuts from a single pine species, and this identity was indicated by the vendor. In cases in which we obtained reference samples of the same species from multiple sources, the samples were identified by letters A–C. The International Nut and Dried Fruit Foundation, Reus, Spain, generously provided samples of pine nuts from *P. armandii* (A), *P. koraiensis* (A), and *P. sibirica* (A). Lawyer Nursery, Inc., Plains, MT, provided seeds from *P. gerardiana* (A); *P. armandii* (B); *P. monophylla*, *P. pumila* (A); *P. sibirica* (B); *P. cembra*, *P. koraiensis* (B); and *P. lambertiana*, *P. edulis*, and *P. wallichiana*. Schumacher Co., Inc., Sandwich, MA, provided seeds from *P. armandii* (C), *P. griffithii*, *P. koraiensis* (C), *P. pumila* (B), *P. tabuliformis*, and *P. yunnanensis*. Wild-crafted pine nuts from *P. edulis*, *P. lambertiana*, and *P. monophylla* were obtained from wildcrops.com.

Korean pine tree seeds were obtained from TreeSeedsforSale.com, Burlington, VT. Seeds from *P. cembroides* (Texas USA) and *P. monophylla* (Nevada USA) were obtained from Kew Gardens by M. Eason, Ladybird Johnson Wildflower Center, University of Texas, Austin, TX, USA. Pine nuts in the shell of *P. armandii*, *P. sibirica*, *P. koraiensis*, and *P. gerardiana* (B) from the Oregon State University (Corvallis, OR, USA) botanical collection were generously provided by Dr. A. Liston. Authenticated seeds from *P. kesiya* (synonym *P. insularis*) were obtained from Botanical Liaisons, Boulder, CO, USA, and authenticated shelled pine nuts of *P. pinea* L. were collected in Sardinia, Italy.

Organic cedar nuts labeled as originating in Russia were obtained from www.FloresFarm.com. Shelled pine nuts labeled as originating in China were purchased at a supermarket in Falls Church, VA, USA. Pine nuts from Pakistan were obtained from Kohenoor International, Hyderabad Sindh, Pakistan. Siberian pine nut oil was obtained from siberiantigernaturals.com and from Igourmet.com. Mediterranean pine nuts were obtained from NutsOnline.com. Other raw pine nuts without species identification were obtained from WholeSalePineNuts.com, NutsOnline.com, and Igourmet.com. Complaint samples from the U.S. market were provided by FDA inspectors. Two samples of pine nuts withdrawn from the Danish market because of consumer complaints were obtained from the Danish Veterinary and Food Administration, Region East, Ringsted, Denmark.

Chemicals. Fatty acid methyl ester (FAME) reference materials (GLC-463, linoleic acid and linolenic acid) were purchased from Nu-Chek Prep (Elysian, MN, USA). Δ5 reference FAMEs were obtained from Cayman Chemical Co. (Ann Arbor, MI, USA) and Larodan Fine Chemicals (Malmo, Sweden). Methanolic hydrochloric acid (HCl/MeOH, 3 N) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Isooctane, methanol (MeOH), and hexane for gas chromatography were purchased from J. T. Baker (Phillisburg, NJ, USA).

Sample Preparation and Methylation. If needed, pine nuts were shelled before grinding. Shelled pine nuts (about 5 g) were ground in a mortar previously washed with MeOH and isooctane. A modification of the method of Destaillats et al.[7] was used to prepare FAMEs as follows:

About 200–250 mg of ground pine nuts was placed in a 20 mL screw cap test tube, and 3 mL of 3 N HCl/MeOH and 2 mL of MeOH were added. The tube was purged with argon and placed in a silicon oil bath (VWR International LLC, Radnor, PA, USA) set at 80 °C for 75 min. After the tube had cooled to room temperature, 3 mL of hexane was added, and the test tube was swirled. The test tube was then filled with a saturated solution of aqueous sodium chloride (NaCl), and the contents were mixed, and then 1 mL of the organic phase was transferred into a new test tube and washed two times with 5 mL of deionized water. The organic phase was filtered through an SPE tube containing anhydrous sodium sulfate and transferred into a 2 mL amber sialinized glass vial. The solvent was removed under a stream of argon at room temperature, and the FAMEs were reconstituted with 1.5 mL of isooctane.

To evaluate the FA composition and botanical identification of known mixtures of pine nuts, we prepared two-component mixtures that contained 50% by weight of each of the reference pine nut species *P. koraiensis* (A), *P. armandii* (A), and *P. sibirica* (A). Mixtures were identified as follows: M1, *P. koraiensis* and *P. armandii*; M2, *P. koraiensis* and *P. sibirica*; and M3, *P. armandii* and *P. sibirica*. FAMEs from the mixed samples were handled as described above.

Gas Chromatographic Analysis with Flame Ionization Detection (GC-FID). GC analyses were performed with a 6890N GC equipped with an FID (Agilent Technologies, Wilmington, DE, USA) and an SLB-IL 111 capillary column (100 m × 0.25 mm, 0.2 μm thickness, Supelco, Bellefonte, PA, USA). Hydrogen was used as carrier gas at 1 mL/min constant flow with the linear velocity of 26 cm/s. The oven temperature was maintained at 168 °C (isothermal elution), and the injection port and FID temperatures were 250 °C. The split ratio was set to 1:100, and a typical injection volume was 1 μL. The injection port liner was a "split only 5SF" from Agilent Technologies. FID additional gases were H_2 at 30 mL/min, air at 400 mL/min, and makeup gas (N_2) at 30 mL/min. The separation time was 30 min.

Separations under the chromatographic conditions described by Destaillats et al.[7] were achieved with the same GC equipped with a fused-silica BPX-70 capillary column (10 m × 0.1 mm i.d., 0.2 μm film thickness; SGE, Melbourne, Australia). Hydrogen was used as carrier gas with a constant flow of 0.6 mL/min. Oven temperature programming was 50 °C isothermal for 1 min, increased to 200 °C at 20 °C/min and then to 250 °C at 50 °C/min. The split ratio was 1800:1, and a typical injection volume was 0.5 μL. The FID was maintained at 250 °C and the injector at 250 °C. Additional FID gases were the same as those listed above.

Each sample was prepared in triplicate, and each replicate was analyzed three times by GC. Means and standard deviations were calculated for each set of injections. FA composition is expressed as weight percent (%, w/w) of total FAs. The identification of FAMEs was made by comparing the FAME retention times with those of commercially available standards (Δ5 FAMEs and GLC-463) and the available literature.

Calculation of the Diagnostic Index (DI). DI values for botanical species identification according to Destaillats et al.[7] were calculated as follows:

$$[(5,\ 9\text{-}18{:}\ 2 + 5,\ 9,\ 12\text{-}18{:}\ 3 + 5,\ 11,\ 14\text{-}20{:}\ 3) / (9\text{-}18{:}\ 1 \text{ and } 11\text{-}18{:}\ 1 + 9,\ 12\text{-}18{:}\ 2 + 11,\ 14\text{-}20{:}\ 2)] \times 10 \quad (1)$$

The 9-18:1 and 11-18:1 FAs were baseline resolved under the chromatographic conditions selected in this study, and DI values were calculated as follows:

$$[(5,\ 9\text{-}18{:}\ 2 + 5,\ 9,\ 12\text{-}18{:}\ 3 + 5,\ 11,\ 14\text{-}20{:}\ 3) / (9\text{-}18{:}\ 1 + 9,\ 12\text{-}18{:}\ 2 + 11,\ 14\text{-}20{:}\ 2)] \times 10 \quad (2)$$

Genetic Differentiation of Pine Nuts. A single seed from each of the reference samples and 3–13 seeds from market samples 21 and 24–26 and from all pine mouth-associated samples were analyzed according to the method of Handy et al.[17]

RESULTS

Chromatographic Separations. The chromatographic separations presented here were obtained using the 100 m SLB-IL 111 capillary column. We also used a 10 m BPX-70 GC column to compare our results with those obtained by Destaillats et al.[7]

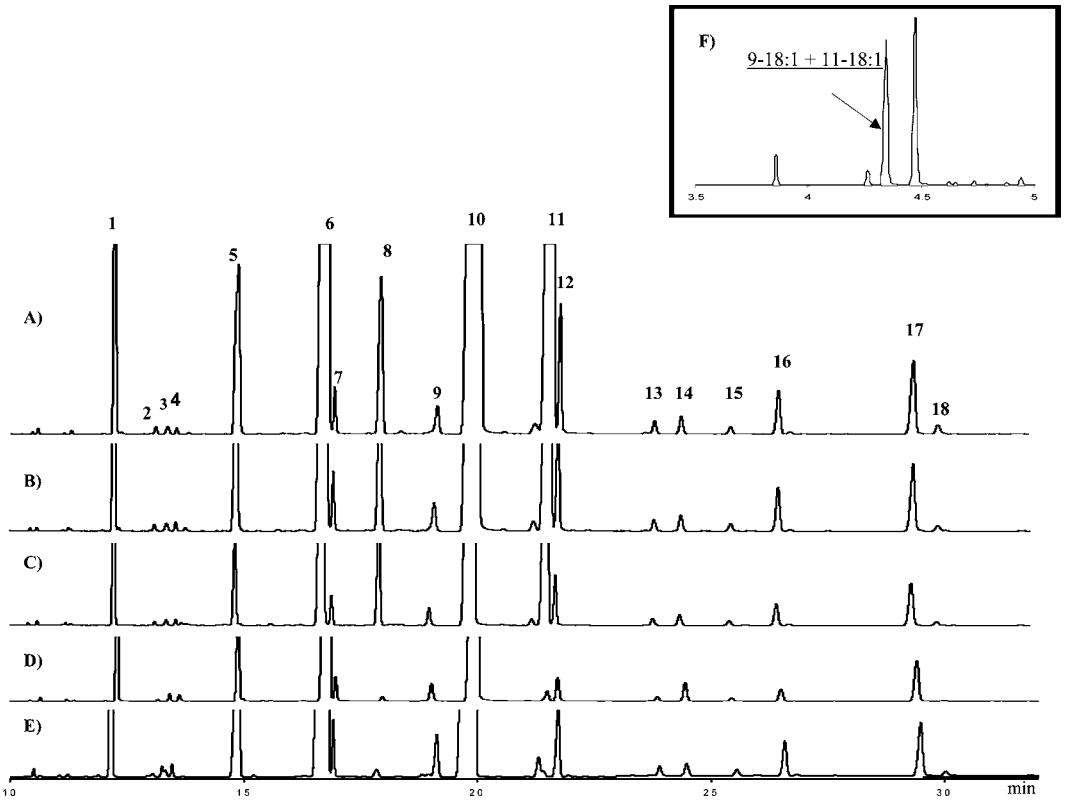

Figure 1. GC-FID chromatogram of pine nut species using the SLB-IL 111 column (Supelco, 100 m × 0.25 mm, 0.2 μm thickness): (A) *P. koraiensis*; (B) *P. armandii*; (C) *P. sibirica*; (D) *P. gerardiana*; (E) *P. pinea* L. (F) GC-FID chromatogram of *P. pinea* L. using a BPX-70 column (10 m × 0.1 mm, 0.2 μm film thickness). Peaks: (1) 16:0; (2) anteiso (ai)-17:0 + 7-16:1; (3) 17:0; (4) 9-16:1; (5) 18:0, (6) 9-18:1; (7) 11-18:1; (8) 5,9-18:2; (9) 20:0; (10) 9,12-18:2; (11) 5,9,12-18:3; (12) 11-20:1; (13) 5,11-20:2; (14) 9,12,15-18:3; (15) 22:0; (16) 11,14-20:2; (17) 5,11,14-20:3; (18) 5,9,12,15-18:4.

Typical GC profiles of FAMEs prepared from *P. koraiensis*, *P. armandii*, *P. sibirica*, *P. gerardiana*, and *P. pinea* L. are shown in Figure 1A–E. The most abundant FAs in these species were 16:0, 18:0, 9-18:1, and 9,12-18:2. All of the unsaturated FAs identified in the analyzed pine nuts showed double bonds only in the *cis* configuration. In addition to these FA, *P. koraiensis*, *P. armandii*, and *P. sibirica* also showed two peaks identified as taxoleic acid (5,9-18:2, peak 8) and pinolenic acid (5,9,12-18:3, peak 11), which were almost completely absent in *P. pinea* L. and *P. gerardiana*. The branched chain FA anteiso-17:0 (peak 2) coeluted with 7-16:1, whereas the two 18:1 FAs, 9-18:1 and 11-18:1 (peaks 6 and 7), were clearly separated. The profile of a sample of *P. pinea* L. (Figure 1F) analyzed using the 10 m BPX-70 column showed that 9-18:1 and 11-18:1 coeluted as a single peak under the fast conditions described by Destaillats et al.,[7] whereas they were resolved into two distinct peaks using the SLB-IL 111 column and the conditions described for this study.

Fatty Acid Composition of Reference Pine Nut Samples. The FA composition and DI value calculated according to eq 2 for reference pine nut species are reported in Table 1. We obtained samples from multiple sources for *P. gerardiana* (1A, 1B), *P. koraiensis* (6A, 6B, 6C), *P. armandii* (7A, 7B, 7C), *P. pumila* (9A, 9B), and *P. sibirica* (10A, 10B). Oleic acid (9-18:1) was found in *P. gerardiana* and *P. pinea* L. at concentrations between 37 and 40% of total FA and at concentrations between 21 and 30% of total FA in *P. koraiensis*, *P. armandii*, and *P. sibirica*.

A total of five Δ5-UPIFA (5,9-18:2; 5,9,12-18:3; 5,11-20:2; 5,11,14-20:3, and 5,9,12,15-18:4) were identified. The major Δ5-UPIFA in *P. koraiensis*, *P. armandii*, and *P. sibirica* were 5,9-18:2 (taxoleic acid, 2–4% of total FA) and 5,9,12-18:3 (pinolenic acid, 13–20% of total FA). Among the remaining Δ5-UIPFA, 5,11,14-20:3 was found at about 1% of total FA in these three species, whereas 5,11-20:2 and 5,9,12,15-18:4 were each found at <0.5% of total FA. In contrast, *P. gerardiana* and *P. pinea* L. contained pinolenic acid at about 0.3% of total FA.

The DI values ranged from 0.05 for *P. gerardiana* to 4.33 for *P. yunnanensis*. The strikingly low DI values for reference samples 1–4 are driven by the relatively high levels of FA 9-18:1 (e.g., 37–46% of total FA) and lower levels of FAs 5,9-18:2, 5,9,12-18:3, and 5,11,14-20:3 compared with corresponding levels in the other samples analyzed.

Pine Nuts Collected in the U.S. Market, Including Those Associated with Pine Mouth. Table 2 lists the DI values, countries of origin if known, and sources of pine nuts and pine nut oils collected from the U.S. market. The DI values of these samples ranged from 0.10 to 3.27 (Table 2). In most cases, particularly among the samples with the higher DI values, the values did not match with any of the reference pine nut samples. This observation indicated that the samples collected in the U.S. market were mixtures of pine nut species.

In addition to the determination of their DI values, the 17 pine mouth-associated samples were subjected to DNA "fingerprinting" for speciation (Table 3). The DI values of the pine mouth-associated samples ranged from 2.41 and 3.37, and most did not match the DIs of the known reference pine nut samples. Genetic analysis showed that 12 of the 17 samples were mixtures of several pine nut species (Table 3). Genetic analysis of four of the market samples (21 and 24–26) also showed them to be mixtures.

Table 1. Fatty Acid Composition and Diagnostic Index (DI) of Reference Pine Nuts[a]

sample	species	16:0	SD	ai-17:0[b]	SD	17:0	SD	9-16:1	SD	18:0	SD
1A	*P. gerardiana*	6.14	0.04	0.07	0.01	0.09	0.01	0.09	0.01	2.32	0.08
1B	*P. gerardiana*	5.63	0.06	0.06	0.01	0.10	0.01	0.10	0.01	2.20	0.09
2	*P. edulis*	6.95	0.03	0.02	0.01	0.08	0.02	0.13	0.00	2.36	0.00
3	*P. monophylla*	7.05	0.00	0.03	0.01	0.09	0.01	0.06	0.02	3.10	0.00
4	*P. pinea* L.	6.20	0.02	0.03	0.00	0.15	0.00	0.10	0.01	3.64	0.11
5	*P. lambertiana*	5.60	0.03	0.06	0.01	0.10	0.02	0.10	0.03	1.60	0.05
6A	*P. koraiensis*	4.71	0.01	0.05	0.00	0.07	0.00	0.06	0.00	2.13	0.03
6B	*P. koraiensis*	5.09	0.00	0.04	0.01	0.07	0.10	0.08	0.01	2.35	0.09
6C	*P. koraiensis*	5.30	0.03	0.04	0.00	0.07	0.00	0.08	0.02	2.05	0.00
7A	*P. armandii*	4.70	0.03	0.04	0.01	0.09	0.01	0.07	0.01	1.88	0.02
7B	*P. armandii*	4.48	0.01	0.04	0.00	0.10	0.00	0.11	0.02	2.19	0.02
7C	*P. armandii*	4.97	0.02	0.06	0.01	0.10	0.04	0.11	0.03	2.43	0.01
8	*P. cembra*	4.66	0.09	0.07	0.00	0.08	0.03	0.07	0.02	2.54	0.03
9A	*P. pumila*	4.07	0.04	0.07	0.02	0.08	0.02	0.07	0.01	2.20	0.09
9B	*P. pumila*	4.00	0.02	0.06	0.01	0.06	0.02	0.08	0.01	2.16	0.01
10A	*P. sibirica*	4.39	0.05	0.07	0.00	0.08	0.01	0.04	0.02	2.49	0.02
10B	*P. sibirica*	4.29	0.01	0.07	0.04	0.07	0.01	0.07	0.00	2.93	0.00
11	*P. griffithii*	4.90	0.01	0.13	0.02	0.08	0.01	0.12	0.01	2.75	0.12
12	*P. wallichiana*	4.67	0.03	0.14	0.07	0.07	0.03	0.12	0.03	2.72	0.02
13	*P. tabuliformis*	5.22	0.02	0.09	0.05	0.09	0.02	0.20	0.03	2.15	0.05
14	*P. kesiya*	5.58	0.01	0.12	0.09	0.16	0.08	0.20	0.01	1.71	0.02
15	*P. yunnanesis*	4.13	0.01	0.13	0.00	0.12	0.01	0.19	0.00	1.72	0.03
sample	species	9-18:1	SD	11-18:1	SD	5,9-18:2	SD	20:0	SD	9,12-18:2	SD
1A	*P. gerardiana*	36.83	0.09	0.86	0.01	0.19	0.04	0.46	0.04	50.87	0.07
1B	*P. gerardiana*	39.52	0.05	0.52	0.00	0.13	0.06	0.40	0.06	49.8	0.08
2	*P. edulis*	44.40	0.02	0.80	0.01	0.15	0.02	0.50	0.05	42.66	0.02
3	*P. monophylla*	46.40	0.03	0.60	0.09	0.51	0.02	0.68	0.04	39.06	0.11
4	*P. pinea* L.	39.47	0.01	0.57	0.02	0.10	0.00	0.60	0.02	44.78	0.03
5	*P. lambertiana*	22.20	0.03	0.71	0.11	2.69	0.08	0.22	0.02	52.50	0.05
6A	*P. koraiensis*	24.79	0.05	0.49	0.06	2.19	0.06	0.38	0.05	46.71	0.09
6B	*P. koraiensis*	29.69	0.03	0.78	0.08	2.26	0.05	0.38	0.04	43.21	0.06
6C	*P. koraiensis*	25.48	0.10	0.71	0.01	2.34	0.05	0.29	0.02	47.03	0.03
7A	*P. armandii*	22.02	0.19	0.49	0.09	3.11	0.02	0.39	0.03	46.92	0.03
7B	*P. armandii*	25.93	0.03	0.62	0.08	3.87	0.03	0.42	0.06	43.95	0.01
7C	*P. armandii*	22.62	0.02	0.78	0.00	3.66	0.06	0.40	0.01	45.56	0.06
8	*P. cembra*	22.62	0.11	0.46	0.01	1.47	0.09	0.31	0.02	45.34	0.05
9A	*P. pumila*	21.99	0.02	0.51	0.01	2.84	0.11	0.27	0.00	45.97	0.04
9B	*P. pumila*	23.18	0.01	0.63	0.02	3.18	0.01	0.24	0.01	44.60	0.03
10A	*P. sibirica*	24.16	0.04	0.37	0.05	1.96	0.02	0.34	0.00	43.78	0.02
10B	*P. sibirica*	21.38	0.09	0.47	0.03	1.92	0.11	0.34	0.06	44.69	0.08
11	*P. griffithii*	16.97	0.12	0.73	0.07	2.34	0.04	0.38	0.01	47.35	0.01
12	*P. wallichiana*	16.55	0.06	0.77	0.01	2.36	0.07	0.39	0.01	46.83	0.11
13	*P. tabuliformis*	20.39	0.05	1.01	0.00	4.12	0.05	0.26	0.02	41.99	0.03
14	*P. kesiya*	19.24	0.01	0.83	0.04	3.08	0.02	0.30	0.03	41.95	0.11
15	*P. yunnanesis*	17.46	0.04	0.92	0.02	2.23	0.02	0.26	0.04	44.27	0.08
sample	species	5,9,12-18:3	SD	20:1	SD	5,11-20:2	SD	9,12,15-18:3	SD	22:0	SD
1A	*P. gerardiana*	0.30	0.04	0.66	0.02	0.05	0.01	0.06	0.03	0.40	0.02
1B	*P. gerardiana*	0.24	0.03	0.63	0.01	0.7	0.02	0.24	0.01	0.13	0.00
2	*P. edulis*	0.38	0.02	0.64	0.02	0.06	0.01	0.19	0.00	0.12	0.00
3	*P. monophylla*	0.80	0.09	0.52	0.08	0.05	0.01	0.27	0.01	0.18	0.02
4	*P. pinea* L.	0.33	0.02	0.86	0.02	0.16	0.02	0.60	0.03	0.12	0.01
5	*P. lambertiana*	10.77	0.01	0.82	0.01	0.03	0.03	0.19	0.01	0.27	0.03
6A	*P. koraiensis*	15.00	0.01	1.25	0.00	0.13	0.02	0.19	0.00	0.10	0.05
6B	*P. koraiensis*	12.38	0.02	1.44	0.01	0.02	0.05	0.14	0.00	0.17	0.04
6C	*P. koraiensis*	13.02	0.02	1.25	0.02	0.16	0.01	0.16	0.02	0.09	0.02
7A	*P. armandii*	16.60	0.00	1.01	0.02	0.16	0.02	0.27	0.03	0.12	0.10
7B	*P. armandii*	14.50	0.01	1.09	0.01	0.21	0.08	0.25	0.03	0.14	0.09
7C	*P. armandii*	15.52	0.01	1.05	0.01	0.03	0.00	0.19	0.00	0.25	0.01
8	*P. cembra*	18.45	0.02	1.31	0.09	0.05	0.02	0.13	0.05	0.33	0.01
9A	*P. pumila*	18.75	0.04	1.13	0.00	0.02	0.00	0.19	0.01	0.21	0.02

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Table 1. continued

sample	species	5,9,12-18:3	SD	20:1	SD	5,11-20:2	SD	9,12,15-18:3	SD	22:0	SD
9B	*P. pumila*	18.71	0.01	1.16	0.01	0.04	0.00	0.18	0.01	0.18	0.01
10A	*P. sibirica*	18.95	0.05	1.14	0.07	0.14	0.01	0.21	0.04	0.08	0.02
10B	*P. sibirica*	19.85	0.03	1.28	0.04	0.04	0.04	0.14	0.05	0.27	0.00
11	*P. griffithii*	20.37	0.01	0.83	0.02	0.01	0.03	0.15	010.C	0.35	0.03
12	*P. wallichiana*	21.19	0.02	0.91	0.00	0.02	0.00	0.17	0.01	0.36	0.01
13	*P. tabuliformis*	16.89	0.01	0.92	0.01	0.03	0.01	0.41	0.04	0.42	0.04
14	*P. kesiya*	17.87	0.03	0.82	0.02	0.48	0.02	0.35	0.00	0.24	0.03
15	*P. yunnanesis*	19.66	0.02	0.83	0.01	0.02	0.01	0.44	0.02	0.50	0.00

sample	species	11,14-20:2	SD	5,11,14-20:3	SD	5,9,12,15-18:4	SD	DI	SD
1A	*P. gerardiana*	0.38	0.01	0.19	0.06			0.08	0.02
1B	*P. gerardiana*	0.18	0.01	0.11	0.01			0.05	0.01
2	*P. edulis*	0.23	0.02	0.29	0.01	0.02	0.00	0.09	0.03
3	*P. monophylla*	0.19	0.02	0.30	0.06	0.30	0.00	0.19	0.02
4	*P. pinea* L.	0.57	0.01	1.85	0.00	0.03	0.13	0.27	0.02
5	*P. lambertiana*	0.84	0.03	1.27	0.01	0.05	0.01	1.95	0.02
6A	*P. koraiensis*	0.59	0.01	1.05	0.04	0.07	0.01	2.53	0.17
6B	*P. koraiensis*	0.76	0.01	1.07	0.00	0.07	0.13	2.13	0.03
6C	*P. koraiensis*	0.69	0.02	1.15	0.01	0.07	0.07	2.26	0.04
7A	*P. armandii*	0.60	0.00	1.36	0.02	0.12	0.02	3.03	0.10
7B	*P. armandii*	0.67	0.02	1.35	0.04	0.08	0.00	2.80	0.09
7C	*P. armandii*	0.76	0.01	1.38	0.03	0.09	0.06	2.98	0.03
8	*P. cembra*	0.80	0.00	1.13	0.01	0.16	0.06	3.06	0.02
9A	*P. pumila*	0.61	0.03	0.89	0.02	0.10	0.04	3.28	0.02
9B	*P. pumila*	0.62	0.01	0.81	0.01	0.09	0.01	3.32	0.01
10A	*P. sibirica*	0.54	0.04	1.09	0.04	0.14	0.01	3.21	0.15
10B	*P. sibirica*	0.86	0.02	1.18	0.10	0.13	0.00	3.43	0.11
11	*P. griffithii*	0.90	0.08	1.44	0.02	0.15	0.01	3.70	0.02
12	*P. wallichiana*	0.93	0.04	1.56	0.01	0.18	0.04	3.90	0.01
13	*P. tabuliformis*	1.12	0.01	4.27	0.03	0.40	0.06	3.98	0.02
14	*P. kesiya*	1.12	0.02	5.44	0.01	0.44	0.01	4.24	0.04
15	*P. yunnanesis*	1.21	0.03	5.35	0.02	0.50	0.03	4.33	0.02

[a]Fatty acid composition is expressed as wt % of total fatty acids. Pine nuts of the same species from multiple sources are indicated by letters. Values are the means ± SD of results of triplicate injections of each of three fatty acid methyl ester preparations per sample. The names of all reference pine nuts are exactly as provided by the supplier. *P. griffithii* is a synonym for *P. wallichiana* and, therefore, these two are not different species. DI = [(5,9-18:2 + 5,9,12-18:3 + 5,11,14-20:3)/(9-18:1 + 9,12-18:2 + 11,14-20:2)] × 10. [b]ai-17:0 and 7-16:1.

Table 2. Sources of Pine Nuts Collected from the U.S. Market[a]

sample	pine nuts	country of origin	distributor	DI	SD
16	pinon nuts (Indian nuts)	?	online store	0.10	0.03
17	pine nuts	Pakistan (vendor)	wholesaler	0.12	0.07
18	pine nuts	?	online store	0.21	0.02
19	Mediterranean pine nuts (pignolias)	?	online store	0.25	0.03
20	pine nuts (pignolias)	?	online store	2.41	0.02
21	pine nuts (pignolias)	?	online store	2.44	0.02
22	pine nuts	?	online store	2.55	0.03
23	pine nuts	China (product label)	retailer	2.60	0.01
24	organic pine nuts	?	online store	2.67	0.02
25	organic pine nuts	?	online store	2.78	0.03
26	pine nuts	?	online store	3.04	0.01
27	pine nut oil	Siberia (product label)	online store	3.15	0.01
28	cedar nuts	Russia (product label)	online store	3.27	0.04

[a]Pine nuts were collected from retail and wholesale distributors and from online stores. Country of origin information, if available, was provided by vendors or listed in product labels. Values are the mean ± SD of results of triplicate injections of each of three fatty acid methyl ester preparations per sample. A question mark (?) indicates that the country of origin was not known. DI = [(5,9-18:2 + 5,9,12-18:3 + 5,11,14-20:3)/(9-18:1 + 9,12-18:2 + 11,14-20:2)] × 10.

The FA composition of selected commercial samples collected from the U.S. market is reported in Table 4. Samples M1, M2, and M3 are mixtures of 50% by weight (w/w) of *P. koraiensis* and *P. armandii* (M1), *P. koraiensis* and *P. sibirica* (M2), and *P. armandii* and *P. sibirica* (M3). Samples 16−19 are pine nuts with DI values of <0.5, and samples 21 and 24−26 are pine nuts with higher DI values (>2.44). Samples 35 and 42 are pine nut samples with high DI values that were associated with

Table 3. Genetic Assessment and Diagnostic Index of Pine Nuts from the U.S. Market[a]

sample	DI	SD	genetic assessment
Pine Nut Samples from the U.S. Market Associated with Pine Mouth			
29	2.41	0.02	mix of *P. koraiensis* and *P. armandii*
30	2.43	0.01	mix of *P. koraiensis* and *P. armandii*
31	2.50	0.02	mix of *P. koraiensis* and *P. armandii*
32	2.55	0.02	mix of *P. koraiensis* and *P. armandii*
33	2.62	0.06	mix of *P. armandii* and *P. gerardiana*
34	2.67	0.01	mix of *P. koraiensis* and *P. armandii*
35	2.79	0.02	mix of *P. koraiensis* and *P. armandii*
36	2.91	0.03	*P. armandii*
37	2.96	0.01	*P. armandii*
38	3.00	0.04	mix of *P. cembra/sibirica* and *P. armandii*
39	3.00	0.03	*P. armandii*
40	3.00	0.01	*P. armandii*
41	3.09	0.05	mix of *P. cembra/sibirica* and *P. armandii*
42	3.10	0.03	mix of *P. koraiensis*, *P. cembra/sibirica*, and *P. armandii*
43	3.18	0.02	mix of *P. cembra/sibirica*, *P. pumila*, and *P. armandii*
44	3.16	0.02	*P. armandii*
45	3.37	0.03	mix of *P. cembra/sibirica* and *P. armandii*
Pine Nut Samples from the U.S. Market			
21	2.44	0.02	mix of *P. koraiensis* and *P. armandii*
24	2.67	0.02	mix of *P. koraiensis* and *P. armandii*
25	2.78	0.03	mix of *P. koraiensis* and *P. armandii*
26	3.04	0.01	mix of *P. cembra/siberica* and *P. armandii*

[a]Species assignments were made as described under Materials and Methods. Samples 21, 24−26, and 29−43 were obtained from the U.S. market, and samples 44 and 45 were provided by the Danish Veterinary and Food Administration, Region East, Ringsted, Denmark. Values are the mean ± SD of results of triplicate injections of each of two or three fatty acid methyl ester preparations per sample. DI = [(5,9-18:2 + 5,9,12-18:3 + 5,11,14-20:3)/(9-18:1 + 9,12-18:2 + 11,14-20:2)] × 10.

pine mouth. The DI of the mixture of *P. koraiensis* + *P. sibirica* was found to be 2.90 ± 0.03, which is close to that of the reference *P. armandii* (3.03 ± 0.10). For the three laboratory-prepared mixtures M1, M2, and M3, the DI value was lower than that of the reference component species with the higher DI value. Genetic analysis of samples 21, 24−26, 35, and 42 confirmed that they were mixtures of several species. The FA compositions of all of these mixtures were indeterminant (i.e., they did not match a FA profile and DI value of a reference species and thus did not allow a specific species assignment to be made).

DISCUSSION

Chromatographic Separation. For decades, FA analysis by GC has been used as a relatively rapid and simple fingerprinting method to determine the origin of oils and fats. Recently, such analysis has been applied to the taxonomy of conifers. The FA composition of conifer seeds differs according to genus, subgenus, section, and subsection and thus can be used as a taxonomic marker.[11,18−21]

Delmonte et al.[22] recently provided a detailed description of the separation characteristics of the SLB-IL 111 column for FAMEs. The ionic liquid SLB-IL 111 is a fused-silica capillary column capable of providing an enhanced separation of unsaturated FAMEs compared to the highly polar cyanopropyl siloxane columns currently used for FAME analysis (CP-Sil 88, SP 2560).[22] The isothermal elution temperature of 168 °C was selected because it provided the most balanced compromise for the separation of mono- and polyunsaturated FAs in fats and oils, including those found in pine nuts.[20] All characteristic Δ5-UPIFAs (peaks 8, 11, 13, 17, and 18) were separated without coelutions.

Destaillats et al.[7] proposed separating the FAMEs prepared from pine nuts by fast GC using a 10 m BPX-70 column and calculating DI values using eq 1. The separation under the conditions Destaillats et al.[7] recommended was rapid and did not fully resolve 9-18:1 and 11-18:1. The minor content of 11-18:1 was included in the calculation of the DI[7] regardless of the fact that it is not a precursor of any of the Δ5-UPIFAs quantified. The SLB-IL 111 column operated under the conditions described in this study separated the 9- and 11-18:1 FAs and made possible the calculation of a more accurate DI relating the Δ5-UPIFA and their metabolic precursors. Previous papers in which Δ5-UPIFAs were analyzed also used columns and conditions that resolved 9-18:1 and 11-18:1 FAs. Separation of these two isomers has been reported using a 50 m CP-Sil 88 column,[23] a 50 m BPX-70 column,[14] and a 30 m PEG column.[11] However, Nasri et al.[24] did not report the separation of 9-18:1 and 11-18:1 FAs on a CP WAX 52 CB (50 m) column.

Wolff et al.[14] identified 9-18:1 as the metabolic precursor of taxoleic acid (5,9-18:2) and, thus, only this isomer should be included in the calculation of the DI value. The exclusion of the 11-18:1 isomer from the equation proposed by Destaillats et al.[7] generally resulted in slightly higher DI values because the denominator was slightly reduced. Whereas the separation of the two isomers 9-18:1 and 11-18:1 might not appear to be important for calculation of the DI value, it has considerable significance when studies are conducted on the biosynthesis of Δ5-UPIFAs. Thus, the use of GC conditions capable of separating these two FAs is mandatory for such studies.

On the basis of our previous work utilizing the 100 m ionic liquid SLB-IL 111 GC column, we considered that the column might provide an enhanced separation of FAs of possible significance for pine nut speciation, which might have coeluted on columns used in previous studies. Despite the improved separations achieved with the SLB-IL 111 column, our results did not reveal a more unique FA profile for individual pine nut species that might assist in determining their botanical origin. In the case of *P. armandii*,[7,25] we did not find any new or characteristic differences in the FA profile.

Pine Nut Samples Collected from the U.S. Market, Including Those Associated with Pine Mouth. We demonstrated that more accurate DI values can be obtained by use of the SBL-IL 111 column. Using the SLB-IL 111 column, we analyzed a total of 45 pine nut samples and calculated the DI according to eq 2 (Tables 2−4). The DI values for many of the market samples and pine mouth-associated samples were indeterminate. These findings suggested that many of the pine nut samples were mixtures of several species.

The deliberate mixing of known pine nuts (samples M1, M2, and M3) results in different FA distributions and, hence, different DI values, which themselves can lead to an inaccurate botanical identification. In analyzing our pine nut mixes, we obtained DI values close to, or different from, that of *P. armandii* (Table 4). The 50% by weight (w/w) mixing of two species not associated with pine mouth (i.e., *P. koraiensis* and *P. sibirica*) provided an FA profile and DI value similar to those of *P. armandii*.

Table 4. Fatty Acid Composition and Diagnostic Index (DI) of Mixed Pine Nut Samples[a]

sample	16:0	SD	ai-17:0	SD	17:0	SD	9-16:1	SD	18:0	SD	9-18:1	SD	11-18:1	SD	5,9:18:2	SD	20:0	SD
M1	4.67	0.02	0.05	0.02	0.07	0.01	0.05	0.01	2.35	0.00	24.94	0.02	0.43	0.01	2.09	0.01	0.40	0.01
M2	4.47	0.02	0.04	0.05	0.07	0.01	0.04	0.00	2.54	0.01	24.44	0.02	0.39	0.00	2.12	0.00	0.42	0.02
M3	4.76	0.01	0.05	0.01	0.08	0.02	0.05	0.03	2.42	0.01	22.90	0.01	0.40	0.02	2.25	0.00	0.40	0.01
16	7.15	0.01	0.02	0.04	0.05	0.13	0.18	0.01	2.35	0.04	44.83	0.00	0.63	0.03	0.17	0.02	0.46	0.03
17	5.67	0.00	0.08	0.01	0.10	0.06	0.12	0.02	2.06	0.00	38.50	0.02	0.66	0.01	0.28	0.01	0.50	0.01
18	7.17	0.01	0.06	0.07	0.09	0.04	0.10	0.11	2.76	0.02	46.08	0.01	0.51	0.01	0.55	0.03	0.60	0.02
19	5.87	0.11	0.03	0.01	0.15	0.03	0.10	0.02	3.73	0.03	36.50	0.02	0.47	0.02	0.12	0.02	0.63	0.04
21	4.78	0.02	0.04	0.02	0.07	0.00	0.06	0.00	2.23	0.01	25.84	0.02	0.48	0.11	2.33	0.02	0.37	0.03
24	4.40	0.01	0.03	0.01	0.76	0.04	0.60	0.03	2.04	0.01	24.13	0.01	0.45	0.10	2.36	0.01	0.37	0.00
25	4.53	0.03	0.04	0.01	0.08	0.06	0.06	0.04	2.12	0.03	23.63	0.01	0.44	0.00	2.42	0.03	0.35	0.01
26	5.88	0.10	0.06	0.08	0.05	0.02	0.05	0.02	2.67	0.06	23.54	0.02	0.53	0.06	2.99	0.00	0.35	0.00
35	5.41	0.02	0.05	0.01	0.04	0.01	0.04	0.03	2.36	0.02	24.68	0.01	0.53	0.03	3.20	0.00	0.37	0.04
42	4.89	0.00	0.06	0.01	0.09	0.00	0.05	0.01	2.74	0.02	22.74	0.02	0.49	0.02	2.57	0.03	0.40	0.02

sample	9,12-18:2	SD	5,9,12-18:3	SD	20:1	SD	5,11-20:2	SD	9,12,15-18:3	SD	22:0	SD	11,14-20:2	SD	5,11,14-20:3	SD	5,9,12,15-18:4	SD
M1	45.21	0.04	16.21	0.01	1.22	0.00	0.14	0.02	0.20	0.01	0.10	0.01	0.65	0.00	1.11	0.03	0.09	0.01
M2	44.99	0.02	17.14	0.06	1.17	0.00	0.14	0.00	0.20	0.03	0.09	0.06	0.60	0.00	1.04	0.03	0.10	0.01
M3	45.34	0.00	17.80	0.02	1.07	0.01	0.15	0.02	0.23	0.01	0.11	0.02	0.63	0.01	1.22	0.01	0.12	0.00
16	42.17	0.01	0.43	0.00	0.61	0.04	0.05	0.01	0.19	0.00	0.12	0.10	0.24	0.04	0.30	0.02	0.01	0.03
17	49.45	0.03	0.48	0.02	0.68	0.03	0.28	0.07	0.09	0.00	0.52	0.00	0.19	0.03	0.30	0.02		
18	39.42	0.12	0.91	0.03	0.60	0.02	0.05	0.03	0.32	0.02	0.18	0.01	0.21	0.02	0.34	0.02	0.04	0.00
19	48.22	0.09	0.37	0.00	0.70	0.01	0.14	0.00	0.64	0.04	0.15	0.02	0.54	0.02	1.62	0.03	0.04	0.00
21	45.93	0.05	14.16	0.02	1.32	0.01	0.15	0.02	0.17	0.01	0.11	0.02	0.73	0.04	1.22	0.00	0.97	0.03
24	46.81	0.02	15.44	0.01	1.21	0.00	0.16	0.01	0.19	0.02	0.13	0.07	0.70	0.01	1.32	0.00	0.11	0.02
25	46.54	0.01	15.96	0.03	1.19	0.03	0.16	0.00	0.19	0.00	0.12	0.00	0.70	0.01	1.33	0.01	0.11	0.01
26	43.76	0.02	16.62	0.02	0.93	0.01	0.66	0.03	0.15	0.01	0.25	0.00	1.22	0.06	1.22	0.01	—	—
35	43.96	0.00	14.93	0.01	1.02	0.02	0.70	0.02	0.15	0.00	0.21	0.01	0.75	0.04	1.26	0.01	0.11	0.02
42	44.41	0.03	17.35	0.01	1.08	0.00	0.68	0.02	0.25	0.01	0.09	0.01	0.62	0.02	1.18	0.03	0.14	0.00

sample	M1	M2	M3	16	17	18	19	21	24	25	26	35	42
DI	2.76	2.90	3.09	0.10	0.12	0.21	0.25	2.44	2.67	2.78	3.04	2.79	3.10
SD	0.03	0.03	0.05	0.03	0.07	0.02	0.03	0.02	0.02	0.03	0.10	0.02	0.03

[a]Fatty acid composition is expressed as wt % of total fatty acids. Values are the mean ± SD of results of triplicate injections of each of three fatty acid methyl ester preparations per sample. M1, M2, and M3 are 50% by weight (w/w) mixtures of *P. koraiensis* (A) + *P. armandii* (A); *P. koraiensis* (A) + *P. sibirica* (A) and *P. armandii* (A) + *P. sibirica* (A), respectively. Samples 16−19, 21, 24−26, 35, and 42 are samples from the U.S. market. Samples 35 and 42 were associated with "pine mouth". DI = [(5,9-18:2 + 5,9,12-18:3 + 5,11,14-20:3)/(9-18:1 + 9,12-18:2 + 11,14-20:2)] × 10.

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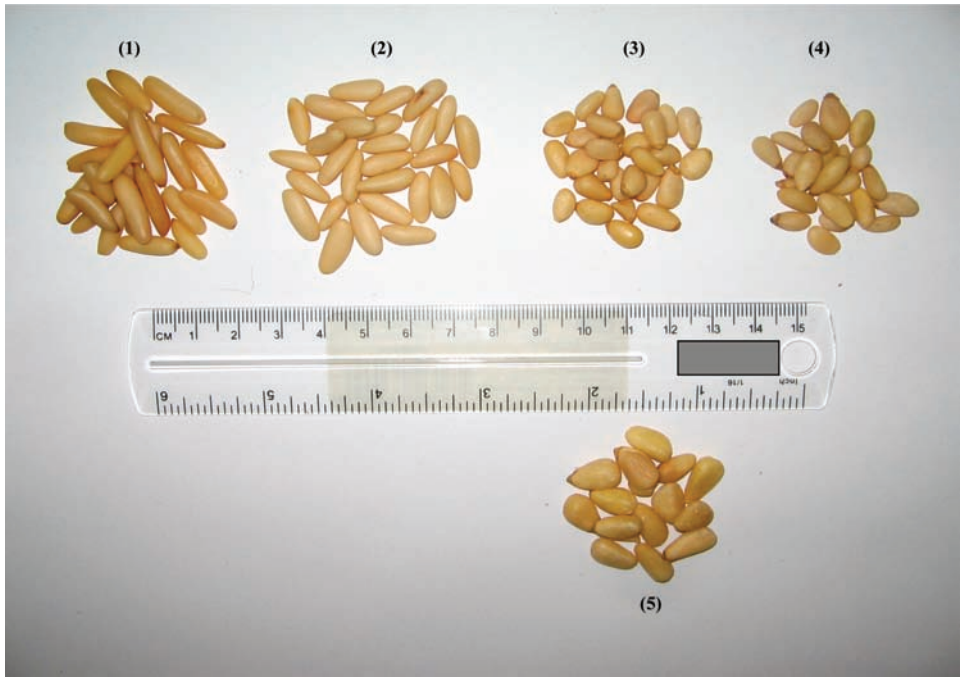

Figure 2. Morphological characteristics of reference pine nuts: (1) *P. gerardiana*; (2) *P. pinea* L.; (3) *P. sibirica*; (4) *P. armandii*; (5) *P. koraiensis*.

There are many varieties of Coniferophytinae,[19,26,27] and the size, shape, and length of their seeds can vary significantly. In the work of Destaillats et al.,[7] when the FA composition and the DI value of a particular pine nut sample did not match those of reference *Pinus* specimens, a visual examination of the nuts was undertaken. In several cases, physical separation or sorting of pine nuts on the basis of their morphology (length, diameter, etc.), followed by reanalysis, revealed the presence of more than one *Pinus* species. One sample was found to consist of a mixture of nuts from *P. koraiensis* and *P. armandii*. Another sample with a DI value close to those of *P. sibirica* and *P. massoniana* was determined to be *P. massoniana* on the basis of its overall FA profile and country of origin stated on the package.[7] Neither *P. armandii* nor *P. massoniana* is listed by the FAO[3] as among the species of pine nuts traditionally used for human consumption. In a further study, Destaillats et al.[28] analyzed 17 samples from consumers who reported dysgeusia following consumption of pine nuts. *P. armandii* nuts were found in all samples, either in pure form or mixed with nuts of *P. koraiensis*.

The preselection of pine nuts on the basis of their morphological characteristics[7] may be neither efficient nor accurate. In Figure 2, we compared the morphological characteristics of five species of reference pine nuts (*P. gerardiana*, *P. pinea* L., *P. sibirica*, *P. armandii*, and *P. koraiensis*). On the basis of the overall length and shape, we observed that seeds of *P. gerardiana* and *P. pinea* L. (Figure 2, seed groups 1 and 2, respectively) were difficult to distinguish, as were seeds of *P. sibirica* and *P. armandii* (Figure 2, seed groups 3 and 4, respectively). The *P. koraiensis* nut is larger and has some differences in shape that distinguish it from the other four species (Figure 2, seed group 5).

Apparent morphological differences become increasingly difficult to discern when large quantities of pine nuts are mixed in unknown ratios (e.g., hundreds or thousands of pounds). A determination of whether mixtures of seeds should be visually sorted becomes even more problematic because in the bulk commercial trade, pine nuts from the same species are marketed on the basis of their size,[29] and pine nut sellers from overseas usually grade pine nuts by the number of kernels per 100 g of seeds (e.g., 650−750 pieces/100 g to 1500−1700 pieces/100 g).[30] On the basis of such considerations, we conclude that selection or sorting prior to the GC analysis is likely to be of little value, and we did not attempt to make a selection of seeds based on morphology prior to analysis.

Characterization by a DNA-Based Method. DNA sequence analysis is a powerful biological tool to identify different species of plants.[31] Because of the complexities associated with identifying pine nut mixtures based on DI values, Handy et al.[17] developed a DNA-based method that more clearly identifies the presence of different *Pinus* species in samples associated with pine mouth and other commercially available pine nut samples. Handy et al.'s[17] objective was to develop a more definitive method for differentiating pine nuts in response to reports that *P. armandii* was associated with pine mouth syndrome.[9,25,28]

On the basis of the DNA analysis, all of the pine nut samples associated with pine mouth collected by FDA inspectors contained *P. armandii*, and five of them appeared to be pure *P. armandii* with DI values ranging from 2.91 to 3.16. The reference DI value for this species in our work is 2.89 ± 0.10. Twelve of the 17 samples associated with pine mouth were found, by genetic analysis and FA analysis to be mixed samples, with *P. armandii*, *P. gerardiana*, *P. cembra*/*sibirica*, *P. pumila*, and *P. koraensis* among the species identified. Similarly, FA analysis and genetic analysis of four of the market samples, which were not to our knowledge associated with pine mouth, also showed them to be mixtures of *P. koraiensis* and *P. armandii* or *P. cembra*/*sibirica*, and *P. armandii*.

Our studies on the FA composition and DI values of market samples and results of DNA-based analyses indicate that many of the pine nuts available in the United States are mixtures of several species of pine nuts. To our knowledge, this is the first work to provide information on FA composition, DI values, and species identification of pine nuts available in the U.S. market.

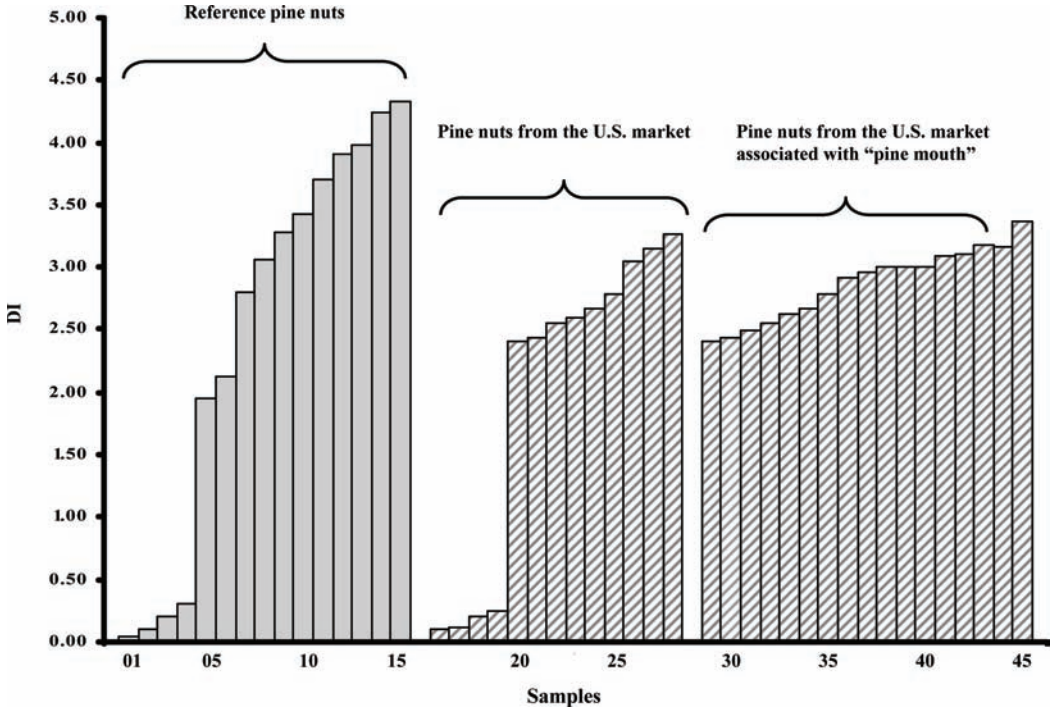

Figure 3. Diagnostic index (DI) of reference pine nuts (samples 1−15) and pine nuts from the U.S. market (samples 16−28), including those from the U.S. market associated with pine mouth (samples 29−43). Samples 44 and 45 are pine nut samples from the Danish market that were associated with pine mouth.

This finding is presented graphically in Figure 3, which shows that there is considerable overlap between the DI values of market samples not associated with pine mouth and those associated with pine mouth. This observation regarding the commercial pine nuts in the U.S. market is in agreement with Sharashkin and Gold,[2] who reported, on the basis of economic rather than analytical considerations, that commercial pine nuts are usually mixtures of different pine nut species.

Of interest is the finding of pine nuts with very low DI values (<0.5) in the U.S. market samples (samples 16−19). On the basis of examination of its FA composition, sample 17, identified by the vendor as originating in Pakistan, may be *P. gerardiana* nuts, which are commonly exported from Pakistan. Sample 19, labeled as Mediterranean pine nuts, may represent a sample of *P. pinea* nuts. Its overall FA composition and high level of 5,11,14-20:3 are consistent with this. The other two samples (16 and 18) may represent pine nuts harvested in the western United States, where *P. monophylla* and *P. edulis* are widely consumed but not yet produced in sufficient quantities for global trade.

Currently Available Methods. Each of the methods we used to determine that pine nuts in the U.S. market consist in general of mixtures has advantages and disadvantages. The FA profile/DI calculation analysis, which can be performed with relatively large sample portions of seeds and does not require presorting before analysis, can clearly show whether DI values match or do not match DI values of reference pine nuts. However, although capable of indicating that a sample of pine nuts is a mixture, the results of the FA profile and calculation of DI values cannot identify the components of the mix. The DNA method, in contrast, provides some species identification but is dependent on the sorting of pine nuts before analysis. We have shown that sorting can be a problematic procedure if morphologically similar species are mixed. Because of the current capabilities of the DNA assay, mixtures may also present a problem when closely genetically related species are mixed. For example, whereas *P. armandii* can be easily resolved from closely related species such as *P. lambertiana*, *P. cembra*, *P. sibirica*, and *P. koraiensis* using the C-D section of the ycfl gene, some other species of pine nuts cannot be differentiated using this region (e.g., *P. yunnanensis/tabuliformis* and *P. cembra/sibirica*).[17] Use of the DNA method would become a problem if, for example, nuts of the edible species *P. sibirica* and *P. cembra* were mixed. In addition, sampling issues due to the use of only one or several seeds would quickly arise if bulk quantities of pine nuts needed to be analyzed.

Köbler et al.[25] recently reported the use of nontargeted 400 MHz ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy to identify *Pinus* species producing nuts that cause taste disturbances. They showed that three groups of pine nuts could be distinguished using principal component analysis. *P. armandii* nuts that were associated with taste disturbances were found in only one of the groups, which, however, also included some *P. armandii* nuts that were not, on the basis of taste testing, associated with taste disturbances.[25] The botanical identification of the pine nuts was based solely on determination of the DI value according to the method of Destaillats et al.[7] Samples with DI values from 2.80 to 3.13 were said to be *P. armandii*. Köbler et al.[25] suggested that their procedure might be used as a means of importation control that would allow the identification of samples suitable for direct clearance while redirecting others for sensory analysis (i.e., organoleptic testing by qualified assessors).

Although the methods currently available, including morphological examination, FA analysis, DNA testing, or NMR analysis, can each provide some useful information about the identity of pine nuts, none have identified a cause of pine mouth syndrome. Recent data from French poison centers covering more than 3000 cases of bitterness following consumption of pine nuts

showed that a rapid increase occurred in May 2009 and peaked in August 2009. The number of reported cases declined sharply after the peak of almost 700 cases reported per month in August 2009.[9] Currently, there is no information about specific samples that were associated with these 3000 cases (e.g., species identification). An etiological agent for pine mouth has not been identified either for the cases reported in Europe or for those reported in the United States. Suggestions regarding causality range from the possible presence of an unidentified toxin (e.g., a contaminant or a natural constituent) resulting from importation of nonedible *Pinus* species to individual susceptibilities possibly related to polymorphism in the genetic expression of taste function. Until the time that the physiological mechanism of pine mouth is understood, monitoring of cases as well as botanical, biological, and chemical characterization of pine nuts will continue to be important.

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Notes

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ABBREVIATIONS USED

FA(s), fatty acid(s); DI, diagnostic index; Δ5-UPIFA, Δ5-unsaturated polymethylene-interrupted fatty acids; methanol, MeOH; ai, anteiso.

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