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Species determination of pine nuts in commercial samples causing pine nut syndrome

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ABSTRACT

Consumption of pine nuts from the species of *Pinus armandii* has been reported to cause dysgeusia, commonly known as pine mouth, or pine nut syndrome (PNS). However, the number of reports on pine nut consumptions of the different species and PNS is limited. This leaves open the possibility that other pine species than *P. armandii* could be involved in PNS as well. This study investigated 18 samples involved in PNS and received at the Danish Veterinary and Food Administration in 2011 through 2012. Samples were subjected to gas chromatographic analysis of fatty acids. The content of 11 individual fatty acids was used together with the diagnostic index and the sum of Δ5-fatty acids as diagnostic parameters. Diagnostic parameters from samples were then compared to reference material and literature data to determine the species. In a limited number of samples, the diagnostic parameters matched neither our reference materials nor literature data. However, the morphology, the fatty acid analysis, and externally obtained DNA sequencing data suggest a *P. armandii* subspecies or a variety. With these possible *P. armandii* subspecies, *P. armandii* was identified in all analyzed samples. The application of principal component analysis (PCA) to the data set showed a satisfactory separation of the majority of the 13 pine species included in the study.

1. Introduction

Pine nut related dysgeusia, now referred to as pine mouth or pine nut syndrome (PNS), was first described in 2001 (Mostin, 2001). In 2010 reports showed up in the literature again, one reporting around 3000 cases in France in 2009 (Flesch et al., 2011). Complaints of pine nut related dysgeusia increased from 2008, peaked in 2009 (Flesch et al., 2011) and in 2011 (Kwegyir-Afful et al., 2013), and then decreased in 2012. The symptoms of PNS are characterized by a constant bitter or metallic taste, often intensified by the ingestion of certain foods (Ballin, 2012; Flesch et al., 2011; Kwegyir-Afful et al., 2013). These symptoms appear 1–2 days after ingestion, resolve within 14 days, and show no spontaneous relapse or other side effects within a year (Ballin, 2012). A large investigation based on a questionnaire showed that the bitter or metallic taste lasted for 9.3 ± 4.8 days ($n = 57$) (Kwegyir-Afful et al., 2013). The causative agent is still unknown and symptoms have, so far, been limited to ingestion of pine nuts from the species of *Pinus armandii* (Destaillats, Cruz-Hernandez, Giuffrida, & Dionisi, 2010; Destaillats et al., 2011; Köbler et al., 2011; Zonneveld, 2011).

P. armandii is one out of more than 100 different *Pinus* species that exist worldwide. According to Food and Agriculture Organization of the United Nations (FAO) http://www.fao.org/docrep/X0453E/X0453e12.htm, 29 of these species produce edible nuts; not all are used commercially. The most common edible pine nuts belong to the species of *Pinus koraiensis*, *Pinus sibirica*, and to a lesser extent *Pinus gerardiana*, *Pinus pinea*, *Pinus pumila*, *Pinus tabuliformis*, *Pinus wallichiana* (formerly known as *Pinus griffithii*), and *Pinus yunnanensis*. Common inedible pine nuts belong to the species of *P. armandii* and *Pinus massoniana*. The genetic variation of *P. armandii* within a limited geographic Chinese area (Chen & Chen, 2011) suggests that different subspecies or varieties might end up as a commodity for international trade. According to The International Plant Names Index (IPNI, 2012), the recognized 8 subspecies and varieties of *P. armandii* include *Pinus armandii var. amamiana*, *P. armandii* Franch (*Pinus armandii var. armandii*), *Pinus armandii var. dabeshanensis*, *Pinus armandii var. farjonii*, *Pinus armandii var. fenzeliana*, *Pinus armandii var. mastersiana*, *Pinus armandii* subsp. *mastersiana*, and *Pinus armandii* subsp. *yuana*. Unfortunately, most

Abbreviations: DI, diagnostic index; FA, fatty acid(s); GC, gas chromatography; NMR, nuclear magnetic resonance; PCA, principal component analysis; PNS, pine nut syndrome; RM, reference material; S, sample.

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commercial pine nuts are not labeled with its subspecies or variety, and it is therefore difficult to obtain reference material for subspecies comparison.

The inedible pine nuts from *P. armandii* are commercialized for animal feed production and industrial purposes. In Europe and the US, *P. armandii* batches have within recent years been sold labeled as *P. sibirica* or as other edible species. Efficient control of pine nut species is, therefore, a focal point in preventing future problems with mislabeling and potential cases of PNS.

DNA sequencing (Handy et al., 2011) and nuclear magnetic resonance (NMR) has been applied to pine speciation with success. NMR has been coupled to principal component analysis (PCA), which is an effective tool in handling large and complicated data sets (Köbler et al., 2011). Köbler et al. (2011) presented a fast screening method that was able to discriminating groups of species and exclude nuts involved in PNS (Köbler et al., 2011). Gas chromatographic (GC) analysis of FAs (FA) (Wolff, Pedrono, Pasquier, & Marpeau, 2000) is another speciation strategy, especially in less equipped laboratories. A review of the FA content of 144 *Pinus* species (Wolff et al., 2000) has shown species specific FA differences. Seven of these species specific FAs made it possible to calculate a diagnostic index (DI) value capable of differentiating between different *Pinus* species (Destaillats et al., 2010). However, further exploration of the DI showed problems in the analysis of samples with mixed species (Fardin-Kia, Handy, & Rader, 2012). In this study, the homogeneity of pine nut samples involved in PNS was evaluated based on the different species morphology. In cases of mixtures, pine nuts were carefully separated into groups of similar morphology prior to analysis. This study shows that the use of the DI in species determination should be confirmed by additional diagnostic parameters. In addition to the DI value, we extended the diagnostic parameters to include the sum of the Δ5-FAs and analysis of eleven individual FAs (denoted diagnostic FAs). Principal component analysis (PCA) was used to visualize how the FA content enabled pine species discrimination.

2. Materials and methods

2.1. Reference material (RM)

Reference materials (RM) were named RM1–RM13. Species, RM names, origin, and year of crop are presented in Table 1. *P. armandii* (RM1), *P. koraiensis* (RM5A), and *P. sibirica* (RM9) were kindly gifted from the International Nut and Dried Fruit Council, Spain (INC).

Table 1
Identity of reference samples, their origin and year of harvest.

Species	Name	Origin	Year of harvest
P. armandii	RM1	China	2010
P. bungeana	RM2	China	2010
P. densata	RM3	Benxi, China	2009
P. gerardiana	RM4	Kashmir	2009
P. koraiensis	RM5A	China	2010
P. koraiensis	RM5B	Copenhagen, Denmark	2011
P. massoniana	RM6	Lishui, Zhejiang	2009
P. pinea	RM7	Unknown[a]	Unknown[a]
P. pumila	RM8	Kamchatka, Russia	2010
P. sibirica	RM9	China	2010
P. tabuliformis	RM10	China	2009
P. wallichiana	RM11	Kashmir	2010
P. yunnanensis	RM12A	Yuannan province of China	2009
P. yunnanensis	RM12B	China	2011
P. cembra	RM13	Germany	2010/2011[b]

[a] Pine nuts were obtained from a local supermarket with no information on the geographic origin and year of harvest.
[b] A mixture of two different crops.

Pinus densata (RM3), *P. gerardiana* (RM4), *P. massoniana* (RM6), *P. pumila* (RM8), *P. wallichiana* (RM11) (formerly known as *P. griffithii*), and *P. yunnanensis* (RM12A) were purchased from Prime Seeds, Denmark. *P. koraiensis* (RM5B) was kindly gifted from the University of Copenhagen, Arboretum Hørsholm, Denmark. *P. pinea* (RM7) was collected in a store and authenticated through a morphological evaluation and a comparison of the FA content with literature data (Wolff et al., 2000). *P. tabuliformis* (RM10) was purchased from Le Semences du Puy, France. *Pinus bungeana* (RM2), *P. yunnanensis* (RM12B), and *Pinus cembra* (RM13) were purchased from OMC Seeds, Lithuania.

2.2. Chemicals and standards

The FAs myristic acid 14:0, palmitic acid 16:0, palmitoleic acid 16:1n-7, margaric acid 17:0, heptadecanoic acid 17:1n7, stearic acid 18:0, oleic acid 18:1n9, vaccenic acid 18:1n7, linoleic acid 18:2n6, arachidic acid 20:0, eicosenoic acid 20:1n9, linolenic acid 18:3n3, docosadienoic acid 20:2n-6, behenic acid 22:0, and lignoceric acid 24:0 were included in the GLC 87, 606, 546B, 17AA′, 68A standard mixes purchased from Nu Chek Prep, INC., Elysian, MN, USA. Methanol, heptane, and n-heptane (all HPLC grades) were purchased from Rathburn Chemicals Ltd, Walkerburn Scotland. 3N hydrogen chloride in methanol (for GC derivatization) was purchased from Sigma–Aldrich, Denmark.

2.3. Samples

Eighteen samples (S1–S18) were either analyzed as part of a national regulatory control project or on a commercial basis. Commercial samples were analyzed for Danish importers and for the Ministry for Health, the Elderly and Community Care (MHEC), Environmental Health Directorate, Malta. All samples (S1–S18) originated from batches involved in PNS consumer complaints, and samples were received and analyzed from February 2011 through 2012.

2.4. GC instrumentation and chromatographic conditions

Two different gas chromatographic (GC) systems were used. The GC-flame ionization detector (FID) system was used for routine determination of pine species, and the GC-mass spectrometer (MS) system was used to identify FAs that were not included in the FA standard mixes commercially available.

The GC-FID system: A polar SP2560 (Supelco, Sigma–Aldrich, Denmark) or a CPSil88 (Agilent Technologies, Denmark) L × i.d. 100 m × 0.25 mm column with a 0.2 μm film was used on a 6890A GC system equipped with an FID (Agilent Technologies, Denmark). The injector temperature was 250 °C. Oven temperature program: Initial oven temperature at 100 °C, raised to 140 °C at 10 °C/min and held for 3 min, to 180 °C min at 5 °C/min, to 200 °C at 1 °C/min, to 215 °C at 5 °C/min, held for 10 min, and finally raised to 230 °C at 5 °C/min and held for 10 min. A post run for 3 min at 100 °C was included. Inlet pressure of helium carrier gas was 29 Psi, split 60:1, with a 4 mm ID split liner. Injection volume was 0.5 μL with a syringe size of 5 μL. Constant column helium flow was 0.8 ml/min. Detector temperature was 275 °C. Hydrogen flow was 40.0 ml/min, the air flow was 450.0 ml/min, and the helium makeup flow was 45.0 ml/min.

The GC–MS system: The CPSil88 column (dimensions described above) was used on a Saturn GC–MS system (Varian Inc.). Chromatographic conditions were similar as for the GC-FID. The GC–MS operated in the electric ionization (EI) mode and was used to obtain a total ion chromatogram (TIC). Scans were performed from 40 to 500 *m*/*z*, with a multiplier offset at 200 V, and a scan time of 0.740 s.