

A novel real-time polymerase chain reaction method for the detection of pecan nuts in food

B. Brežná · T. Kuchta

Received: 7 December 2006 / Revised: 16 March 2007 / Accepted: 26 March 2007 / Published online: 25 April 2007
© Springer-Verlag 2007

Abstract A real-time PCR-based method for the detection of the pecan (*Carya illinoensis*) component in food is described. The method consists of DNA isolation by chaotropic solid phase extraction and the subsequent PCR with pecan-specific primers and a TaqMan fluorescent probe. The primers and the probe are targeted to the putative gene for allergenic vicilin-like seed storage protein of pecan. The method was positive for 10 pecan varieties and negative for all other tested plant materials used in food industry, including walnut. The intrinsic detection limit of the method was 1 pg pecan DNA which corresponds to 1.2 haploid genome copies. Using a series of model pastry samples with defined pecan contents, a practical detection limit of 0.01% (w/w) pecan was estimated. Practical applicability of the PCR method was tested by the analysis of 13 food samples; no discrepancies between the declared and detected pecan contents were found. The presented PCR method is useful for sensitive and selective detection of pecans in food samples and can be performed in one working day.

Keywords Pecan · Allergen · Polymerase chain reaction (PCR) · Food · Confectionery

Introduction

Pecan nuts, the fruits of the pecan tree (*Carya illinoensis*) are edible tree nuts native to North America. In USA, they

are widely used in bakery products as a basic component of nut fillings in various cakes, in a range of confectionery products like filled chocolates, in cereal müsli mixtures and in other food products. In Europe, their use is less frequent at the present time and is mostly limited to gourmet products. Pecans may be a threat to some consumers since they contain allergens and consumption of food products containing pecans may cause severe allergy in a part of the population [1, 2]. In response to this fact, the recently introduced European legislation requires labelling of food products in respect to the contents of pecans [3]. To enforce this legislation, methods for analysis of pecan content in food are necessary. Currently, there is no published method available for the detection of pecans in food products. Although several food analytical laboratories have reported that PCR-based tests for pecan detection are available on request [4, 5], the data necessary for the reproduction of these methods were not published, nor were the genes disclosed, to which the PCR methods are targeted. Here we present a method for the specific detection of pecan DNA by real-time PCR, which is a technology becoming widely used for the detection of allergenic components in food [6].

Materials and methods

Plant and food materials

Leaves of various pecan (*Carya illinoensis*) varieties were obtained from Dr. L. J. Grauke, USDA, ARS Pecan Breeding, Sommerville TX, USA. Pecan nuts were provided by Dr. C. E. Cerniglia, National Center for Toxicological Research, US FDA, Jefferson, Arkansas, USA. Nuts of various walnut varieties were obtained from Central

B. Brežná (✉) · T. Kuchta
Department of Microbiology and Molecular Biology,
Food Research Institute, Priemyselná 4,
P. O. Box 25, 824 75 Bratislava 26, Slovakia
e-mail: brezna@vup.sk



Fig. 1 Region of the pecan putative vicilin-like seed storage protein gene, to which the PCR primers and the probe were designed, and the comparison to the homologous genes of selected agricultural plants. Adapted from [7]. GenBank accession numbers: pecan DQ156215; walnut AF066055; macadamia AF161883; cocoa X62625; sesame

AF240006; hazelnut AF441864. Symbols: Upward arrow—genomic DNA sequence; Degrees sign—mRNA sequence; identity with the genomic DNA was confirmed by sequencing; Asterisk—mRNA sequence

Institute for Supervising and Testing, Brno, Czech Republic. Sprays of non-food species of *Juglans* and *Carya* genera were provided by Dr. I. Štefančík, Forestry Research Institute, Zvolen, Slovakia. Wheat, barley and rye flour as well as potato starch were obtained from Dr. F. Schwägle, Federal Research Centre for Nutrition and Food, Kulmbach, Germany. Other nuts and food samples were obtained from grocery stores. Standard pastry with defined pecan concentration was prepared analogously as previously described for walnut model pastry [7].

DNA extraction

Prior to DNA extraction, samples were homogenized (without prior freezing). Food and leaf samples were ground using mortar and pestle. Wood-containing samples, i.e. nutshells and sprays, were ground to a fine powder using emery (rasp paper). Subsequently, leaf material (100 mg) was processed using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The protocol included lysis, RNase digestion, salt precipitation of the contaminating proteins and polysaccharides and silica-gel-based chaotropic solid phase extraction of the DNA. DNA from food and wood samples (200 mg and 100 mg, respectively) was isolated using the GeneSpin kit (GeneScan, Freiburg, Germany) by a similar procedure,

which, however, includes proteinase K digestion and omits RNase digestion. As an exception, preparation of highly purified pecan seed DNA for the theoretical detection limit determination was performed by GeneSpin procedure modified by adding 2 µl of 100 mg/ml RNase A (Qiagen) to the cleared lysate.

DNA quantitation

DNA was quantitated fluorimetrically using a DNA intercalation dye PicoGreen (Molecular Probes, Leiden, Netherlands). Dilutions of bacteriophage lambda DNA (Fermentas, St Leon-Rot, Germany) were used to construct the calibration line. Fluorescence was measured in a Genios Fluorimeter (Tecan, Grödig bei Salzburg, Austria) at an excitation wavelength of 492 nm and emission wavelength of 520 nm.

For the purposes of theoretical LOD determination, the pecan DNA stock concentration was independently verified by UV spectrometry, using A_{260} for calculation.

Design of the pecan DNA - targeted PCR primers and a probe

Primers and a FAM-labelled, TAMRA-quenched oligonucleotide probe for pecan detection targeted the putative

Table 1 Oligonucleotides used in PCR

Designation	Sequence (5'–3')	Target	Reference
caryaF	ccgcgaagagaaagcagag	Pecan	This article
caryaR	tcattgtctgacctgagtc	Pecan	This article
JugIP	FAM-attgtgcctctgttgcctcttcccg-TAMRA	walnut, pecan	7
TR03	tctgcctatcaacttcgatgta	eukaryotic	8
TR04	aatttgcgcctgctgccttctt	eukaryotic	8
TRPb	FAM-ccgtttctcaggctccctctccggaatgaacc-TAMRA	eukaryotic	9

Table 2 Analysis of the selected species by real-time PCR. C_T values resulting from pecan-specific and universal eukaryotic (amplification control) reactions

Species	C_T pecan-specific	C_T amplif. control
<i>Carya illinoensis</i> “Pecan 1”	27.16	21.05
<i>Carya illinoensis</i> “Pecan 2”	26.35	18.96
<i>Carya illinoensis</i> Curtis ^a	30.14	23.47
<i>Carya illinoensis</i> Desirable ^a	29.60	22.50
<i>Carya illinoensis</i> Elliot ^a	29.86	22.47
<i>Carya illinoensis</i> Major ^a	29.85	22.09
<i>Carya illinoensis</i> Pawnee ^a	30.33	22.09
<i>Carya illinoensis</i> Peruque ^a	29.43	21.98
<i>Carya illinoensis</i> Schley ^a	30.16	22.23
<i>Carya illinoensis</i> Stuart ^a	29.35	23.31
<i>Carya illinoensis</i> Western ^a	29.58	22.95
<i>Carya illinoensis</i> Wichita ^a	29.78	22.74
<i>Carya cordiformis</i> ^a	30.12	23.43
<i>Carya glabra</i> ^a	29.03	22.35
<i>Carya lacciniosa</i> ^a	29.98	23.41
<i>Carya ovata</i> ^a	30.41	22.55
<i>Carya tomentosa</i> ^a	30.04	22.14
<i>Juglans regia</i> “Walnut 1”	–	19.22
<i>Juglans regia</i> “Walnut 2”	–	23.50
<i>Juglans regia</i> Apollo	–	21.00
<i>Juglans regia</i> Buchlov	–	23.30
<i>Juglans regia</i> Jupiter	–	28.55
<i>Juglans regia</i> Lake	–	21.99
<i>Juglans regia</i> Magdon	–	20.80
<i>Juglans regia</i> Mars	–	19.10
<i>Juglans regia</i> Saturn	–	21.83
<i>Juglans regia</i> Seiferdorský	–	18.96
<i>Juglans cinerea</i>	–	24.31
<i>Juglans cordiformis</i>	–	22.99
<i>Juglans mandshurica</i>	–	21.99
<i>Juglans nigra</i>	–	26.58
<i>Juglans sieboldiana</i>	–	22.57
Peanut	–	19.90
Hazelnut	–	18.81
Pistachio	–	19.66
Cashew	–	18.56
Almond	–	19.12
Brazil nut	–	22.62
Macadamia nut	–	14.63
Chestnut	–	16.70
Sunflower	–	15.43
Sesame	–	14.45
Apricot	–	15.57
Peach	–	11.45
Soy	–	17.68
Kidney bean	–	14.83

Table 2 continued

Species	C_T pecan-specific	C_T amplif. control
Maize	–	12.96
Wheat	–	15.01
Barley	–	11.67
Rye	–	11.32
Rice	–	18.07
Potato	–	25.78
Cocoa	–	25.03
Chicken	–	23.94
Pork	–	25.93
Beef	–	25.76
Turkey	–	21.79

^a Samples were adjusted to 0.5 ng per PCR reaction to enable comparison

gene coding for vicilin-like seed storage allergenic protein of pecan (GenBank accession No. DQ156215). The primers CaryaF and CaryaR (Table 1) were designed manually to produce an amplicon 50–100 bp long, to have a T_m between 58 and 60 °C and to be 17–24 bases long. Additionally, the reverse primer was designed in a way to match the DNA region divergent from walnut (Fig. 1). The T_m values were calculated using the Davis, Botstein, Roth Melting Temperature option of the Editseq software (DNASTar, Madison, Wisconsin, USA). The probe was identical to the previously published JugIP probe [7].

Assessment of DNA amplifiability

To check the amplifiability of the isolated DNA, real-time PCR with universal eukaryotic primers and a FAM-labelled, TAMRA-quenched oligonucleotide probe targeted to 18S rRNA gene ([8, 9], Table 1) was performed. Only those DNA samples, which demonstrated PCR amplifiability in this test, were included in subsequent PCR experiments for pecan DNA detection.

Polymerase chain reaction

PCR was performed in a volume of 25 µl. Each reaction contained 5 µl (1–50 ng, unless indicated otherwise) of template DNA, 1.5 U DNA polymerase HotStarTaq (Qiagen, Hilden, Germany), 0.5 U heat-labile uracil DNA glycosylase (Roche, Penzberg, Germany), 1 × PCR buffer II (Applied Biosystems, Foster City, California, USA), 2.5 mmol/l $MgCl_2$, 200 µmol/l dNTP mix with dUTP (Applied Biosystems), 500 nmol/l of forward primer, 500 nmol/l of reverse primer and 200 nmol/l of the probe. PCR was performed in a PTC-200 PCR cycler with an opti-

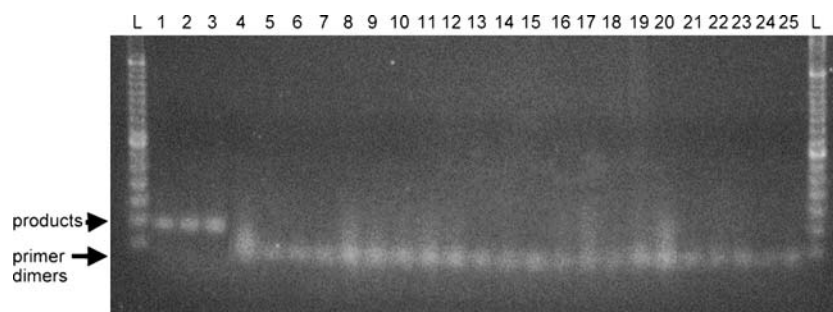
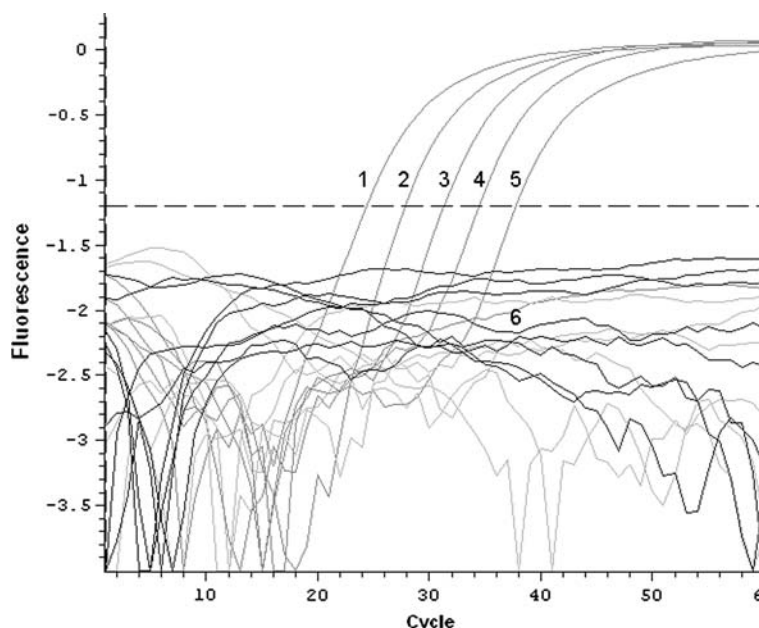


Fig. 2 Agarose gel electrophoresis of DNA from food-relevant species after pecan-specific real-time PCR. L, 50 bp DNA ladder; 1 “pecan 1”, 2 pecan Western, 3 pecan Desirable, 4 “walnut 1”, 5 walnut Apollo, 6 walnut Buchlov, 7 walnut Jupiter, 8 peanut, 9 hazelnut, 10 pistachio,

11 cashew, 12 almond, 13 brazil nut, 14 macadamia, 15 rice, 16 maize, 17 wheat, 18 barley, 19 rye, 20 soybean, 21 pork, 22 beef, 23 chicken, 24 cocoa, 25 no template control

Fig. 3 A record of pecan-specific real-time PCR. Numbered, *medium-gray curves* denote results on decimal dilutions of pecan DNA, i.e. 10 ng (1), 1 ng (2); 0.1 ng (3); 0.01 ng (4); 0.001 ng (5); 0.0001 ng (6). *Dark gray curves* depict results on eight walnut varieties (Apollo, Buchlov, Jupiter, Lake, Magdon, Mars, Saturn, Seiferdorfsky), while *light gray curves* depict peanut, hazelnut, almond, wheat, cocoa and no template control



cal unit Chromo 4 (MJ Research, Waltham, Mass., USA). The PCR programme consisted of the 10 min incubation at laboratory temperature, initial denaturation at 94 °C for 15 min, followed by 60 cycles (however, 50 cycles are sufficient, as demonstrated by the results) of denaturation at 94 °C for 15 s and annealing with polymerization at 60 °C for 60 s. The fluorescence was measured in channel 1 (FAM). The threshold cycle C_T was calculated for individual samples by the internal software of the cycler using the manual threshold setting at a fluorescence value of 0.063.

Results and discussion

DNA sequence of the putative vicilin-like seed storage protein gene of pecan, which was previously sequenced in our laboratory [7], was used for the design of a real-time PCR system for pecan detection in food. To ensure the distinction from walnut, a closely related food-relevant species,

the reverse primer was designed to match the region divergent between these two species (Fig. 1). Unlike walnut, vicilin-like protein genes known from other agricultural plants have a considerably lower homology to pecan and, based on the sequence alignment, cross-reactivity with the pecan-targeted TaqMan system was not probable [7] (Fig. 1).

PCR employing novel primers and the probe targeting the vicilin-like protein gene of pecan was used to analyse DNA isolated from the plant material of pecans (*Carya illinoensis*), other Juglandales and from selected taxonomically unrelated agricultural plants which are widely present in nut-containing food products.

All the pecan samples, which included two independent nut samples and leaves of ten pecan varieties (Western, Stuart, Desirable, Wichita, Pawnee, Schley, Peruque, Majoe, Elliot and Curtis), produced positive results, as well as other *Carya* spp. (*C. cordiformis*, *C. glabra*, *C. laccini-osa* and *C. ovata*). This means that the method was 100% inclusive for the genus *Carya*. The positive detection of

Table 3 Analysis of data points of the theoretical detection limit determination

ng _{ref}	n	C _T avg.	SD (C _T)	linearity range	ng _{calcul.} avg.	RSD (ng _{calcul.})
1.1×10^{-4}	1	38.36	n.a.	—		
3.6×10^{-4}	2	38.52	0.12	—		
1.1×10^{-3}	6	37.72	0.50	+	9.9×10^{-4}	34%
3.6×10^{-3}	6	35.88	1.02	+	4.0×10^{-3}	56%
1.1×10^{-2}	6	33.98	0.52	+	1.2×10^{-2}	34%
1.1×10^{-1}	6	30.75	0.19	+	1.0×10^{-1}	13%
1.1×10^0	6	27.31	0.16	+	1.1×10^0	12%
1.1×10^1	6	23.90	0.11	+	1.1×10^1	9%
3.6×10^1	6	23.20	0.17	—		

ng_{ref}—amount of DNA [ng] added to PCR reaction; n—amount of positive signals out of 6 parallel experiments; C_T avg.—average threshold cycle; SD (C_T)—standard deviation of threshold cycle average; ng_{calcul.} avg.—average of DNA amount-values calculated from raw data points using the regression curve; RSD (ng_{calcul.})—relative standard deviation of ng_{calcul.} average; n.a.—not applicable

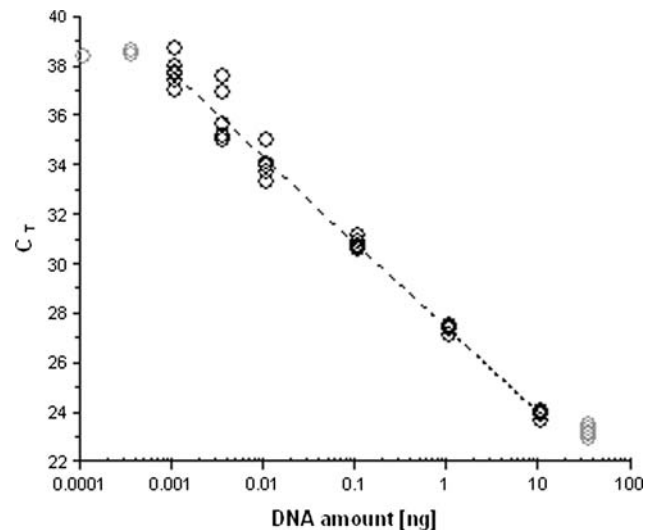
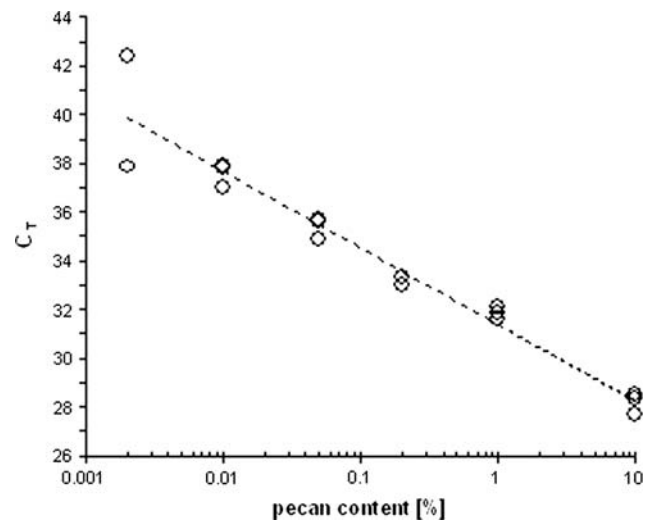
Regression curve between ng_{ref} and C_T avg.:

$$y = -3.415 (\log_{10}x) + 27.39; r^2 = 0.9996$$

Carya species other than *C. illinoensis* is of a little significance for the application of the method to food analysis, since these species are not regularly used in food industry. The method produced negative results with all other tested plant materials (Table 2; Figs. 2, 3), which means the exclusivity of the method for species other than *Carya* was 100 %.

The detection limit of PCR method has been defined as a number of copies, which result in at least 95% of the samples in a positive signal. Using dilutions of the pecan DNA (Figs. 3, 4, Table 3), the intrinsic detection limit of the method was determined as 1 pg, which was the minimal amount of pecan DNA amplified in all parallel reactions. Taking into consideration the molecular weight of the haploid pecan genome, which is 0.83 pg [10], the detection limit of the method corresponds to 1.2 haploid genome copies. However, it is important to note, that this value is tied to best quality stock solution of pecan DNA (the least degraded available and RNase treated) and recently re-calibrated real-time PCR instrument. Using RNA-rich DNA preparations and/or different PCR instruments (not shown) resulted in one order of magnitude higher detection limit (10 pg or 12 copies).

By the analysis of model pastry samples with defined pecan contents, a practical detection limit of 0.01% (w/w) was determined (Fig. 4). A linear relationship between the logarithmic value of pecan concentration and the threshold cycle, C_T was observed (Fig. 5), when the template DNA amount was adjusted to 10 ng per reaction. This observation suggests that the method can be adjusted to be quantitative after standardizing the amount of DNA and after

**Fig. 4** Raw data points of the theoretical detection limit determination of pecan-specific real-time PCR**Fig. 5** Relationship ($y = -3.319 \log_{10}x + 31.21$; $r^2 = 0.947$) between the pecan contents in the model pastry (triplicate samples) and the threshold cycle C_T in real-time PCR. (At 0.002% pecan contents, only two of three parallels were amplified because of low pecan DNA copy number.) The template DNA amount was adjusted to 10 ng per reaction

validation. Less than 0.01% (w/w) pecan content, i.e. 0.002% (w/w), resulted in amplification in some, but not in all replicates (two of three parallels are positive in Fig. 5), and in a greater variability of C_T values. This observation can be explained by a stochastic character of PCR at low template copy numbers.

The developed PCR was applied to the analysis of 13 food products which were declared to contain pecan nuts (6 samples), walnuts (1 sample) or had no declared pecan content but were produced by the companies which process pecans (5 samples). PCR results conformed to the labelling in all the samples (Table 4).

Table 4 Analysis of bakery and confectionery products by real-time PCR. C_T values resulting from pecan – specific and universal eukaryotic (amplification control) reactions

Food sample	Country of origin	Pecan declaration	C_T pecan– specific	C_T amplif. control
Biscuits with caramel and pecans	Scotland	+	30.87	19.79
Biscuits with almonds	Scotland	— ^{a,b}	—	18.00
Biscuits with pecans	France	+	30.15	20.47
Biscuits with butter	France	— ^a	—	19.62
Milk chocolate with pecans	Belgium	+	28.89	22.94
Dark chocolate with hazelnuts	Belgium	— ^{a,b}	—	22.70
Milk chocolate with pecans	France	+	36.83	23.76
Milk chocolate	France	— ^{a,b}	—	25.78
Dark chocolate with hazelnuts	France	— ^{a,b}	—	23.38
Crunchy snack with peanut	France	— ^a	—	22.85
Pralines with caramel and pecans	USA	+	31.21	24.56
Pie with pecan filling	USA	+	27.92	21.40
Milk chocolate with walnuts	Switzerland	—	—	24.11

^a Produced by a company which also processes pecan nuts

^b Declaration “may contain traces of (other) nuts”

As demonstrated, the presented PCR method is useful for sensitive and selective detection of pecans in food samples. The method is relatively straightforward and fast, the analysis can be performed in one working day.

Acknowledgments This research was done in frames of a project “Development of progressive methods and procedures to support quality and safety of food protection and control” of the Slovakian Department of Agriculture. Authors wish to thank Dr. L. J. Grauke, Ph.D., Sommerville, Texas, USA for providing pecan varieties, Dr. C. E. Cerniglia, Ph.D., Jefferson, Arkansas, USA for pecan nut samples, Mgr. T. Jan, Brno, Czech Republic for walnut varieties, and Ing. I. Štefančík, CSc., Zvolen, Slovakia for samples of other *Juglandales*.

References

1. Sicherer SH, Sampson HA (2000) *Curr Opin Pediatr* 12:567–573
2. Roux KH, Teuber SS, Sathe SK (2003) *Int Arch Allergy Immunol* 131:234–244
3. Directive 2003/89/EC of the European Parliament and of the Council of 10 November 2003 amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. *Off J Eur Union* L 308, 15–18
4. Poms RE, Anklam E, Kuhn M (2004) *J AOAC Int* 87:1391–1397
5. Flanagan S (2006) *Food Sci Technol* 20:43–46
6. Pöpping B, Holzhauser T (2004) *Dtsch Lebensmitt-Rundsch* 100:285–293
7. Brežná B, Hudecová L, Kuchta T (2006) *Eur Food Res Technol* 223:373–377
8. Allmann M, Candrian U, Höfelein C, Lüthy J (1993) *Z Lebensm Unters Forsch* 196:248–251
9. Brežná B, Hudecová L, Kuchta T (2005) *Archiv Lebensmittelhyg* 56:112–113
10. Bennett MD, Bhandol P, Leitch IJ (2000) *Ann Bot (Lond)* 86:859–909