JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Omics Technologies Applied to Agriculture and Food

Detection of Peanut Adulteration in Food Samples by NMR Spectroscopy

Caroline Schmitt, Tim Bastek, Alina Stelzer, Tobias Schneider, Markus Fischer, and Thomas Hackl

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.0c01999 • Publication Date (Web): 27 May 2020

Just Accepted

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Detection of Peanut Adulteration in Food Samples by NMR Spectroscopy

Caroline Schmitt[1], *Tim Bastek*[1], *Alina Stelzer*[1,2], *Tobias Schneider*[1], *Markus Fischer*[2] *and Thomas Hackl*[1,2]*

[1]Institute of Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany

[2]HAMBURG SCHOOL OF FOOD SCIENCE - Institute of Food Chemistry, University of Hamburg, Grindelallee 117, 20146 Hamburg, Germany

ABSTRACT

The addition of cheap and also readily available raw materials, such as peanut powder, to visually and chemically similar matrices is a common problem in the food industry. When peanuts are used as adulterant, there is an additional risk of potential health hazard to consumers due to allergy-induced anaphylaxis. In this study, different series of peanut admixtures to visually similar food products, such as powdered hazelnuts, almonds and walnuts, were prepared and analyzed by ^{1}H NMR spectroscopy. For identification, an isolated signal at 3.05 ppm in the ^{1}H NMR spectrum of polar peanut extract was used as an indicator of peanut adulteration. The chemical marker was identified as (2*S*,4*R*)-*N*-methyl-4-hydroxy-L-proline by resynthesis of the compound and use as internal standard. The signal-to-noise ratio and the integral of the signal of the marker can both be used to detect peanut impurities. Overall, an approximate limit of detection of 4% admixtures of peanut in various food products was determined using a 400 MHz spectrometer. In regard to food fraud, we present a viable screening method for detection of economic relevant peanut adulteration.

Keywords: adulteration, peanut, NMR spectroscopy, metabolomics

INTRODUCTION

The adulteration of food products to achieve higher profits is a common problem in the food market.[1] High price differences are an important factor and a potential incentive for food fraud and do not exclusively concern adulterating with the same product, but can also lead to chemically and visually similar admixtures in food.[2] With the increasing global trade of food and the resulting uncertainties, it is necessary to have analytical methods at hand in order to be able to detect adulterations as quickly and easily as possible, but also validly.[3] The most common analytical methods used in recent years include e.g. chromatography and spectroscopy based techniques.[4,5] For example, near-infrared reflectance (NIR) spectroscopy was used to identify purple sweet potato, white sweet potato and their adulterated samples.[5] Furthermore, gas chromatography combined with multivariate data analysis was used to obtain fatty acid fingerprints of almond powder samples adulterated with apricot kernels.[3] There are many studies towards detection of adulterations of different oils, which are often affected by food fraud, whereby a relatively expensive vegetable oil, such as olive oil, is adulterated with low-cost and low-quality oil (e.g. sunflower oil).[6–8]

Nuclear magnetic resonance (NMR) spectroscopy as a non-destructive and relatively rapid method could be an alternative to detect adulteration in food products.[9] Furthermore, NMR spectroscopy shows several advantages such as rich spectral information, the possibility of structure elucidation and fast acquisition times.[10] A relatively new but increasingly important field is the analysis of mixtures of different foods using NMR spectroscopy. For example, ^{1}H NMR spectroscopy was applied to identify the presence of Arabica and Robusta species in coffee.[10,11] Arabica is affected by food fraud, because Robusta with its lower price is used as adulterant.[12] Differences in chemical composition were examined leading to the diterpene 16-*O*-methylcafestol (16-OMC) as a chemical marker in Robusta beans.[10,11] Furthermore, NMR spectroscopy can be used in combination with chemometric algorithms, as Parker *et al.* have shown for the detection of olive oil adulteration with hazelnut oil using a 60 MHz spectrometer.[13] An analytical

protocol for the detection of adulterations of olive oil with hazelnut oil down to 10% using a 600 MHz spectrometer is presented by Mannina *et al.*[8] A new approach in assessment of mixtures by spectral superposition was shown by Bachmann *et al.* for detection of admixtures of hazelnuts from different geographical origin.[14]

For economic reasons it is important to identify food fraud with peanut and verify the quality of affected products.[15] The detection of adulteration of relatively expensive food products, such as almond (*Prunus dulcis* MILL.), with peanut (*Arachis hypogaea*) is difficult due to similarity in chemical composition and beyond the low price and high availability is an incentive for food fraud.[15] Additionally, undeclared admixtures can be life-threatening for peanut allergy sufferers even in smallest concentrations.[16] Peanuts are usually detected by their allergens using immunological techniques, such as enzyme-linked immunosorbent assay (ELISA), DNA based techniques, such as polymerase chain reaction (PCR) and MS based techniques.[16–21] Food fraud also concerns hazelnuts (*Corylus avellana*) and walnuts (*Juglans regia*), which also have a chemical composition similar to that of peanuts and which are used mainly in confectionary and bakery industry as powder, making it even more difficult to distinguish between them.[15,22,23] This problem can be seen in the report of the international investigation operation OPSON VI organized by Europol/INTERPOL which showed that one batch of 1.3 tonnes of roasted chopped hazelnuts were adulterated with 8% peanuts.[24] Using NMR spectroscopy with fast acquisition times has advantages because of qualitative and quantitative detection of adulterant and is therefore a promising alternative method to other already available approaches.[10]

In this study, a novel technique for detecting peanut adulteration was developed using NMR spectroscopy. We analyzed three peanut blends of food products with economic relevance, that are visually and chemically similar when powdered, such as almond, hazelnut and walnut. It was found that the spectrum of the polar extract of peanut at 3.05 ppm shows a universal marker signal associated to (2*S*,4*R*)-*N*-methyl-4-hydroxy-L-proline (1) that can be used to identify peanut additives in these nuts. In

order to assess the applicability as a screening method for qualitative detection in regard to food fraud linear regression models were developed using the calculated signal-to-noise ratio of the marker's signal and the known peanut content of each sample in the mixture series. By measuring twelve different peanut samples the biological variance of the marker was estimated. To validate the models obtained and verify the limitations due to natural biological variance, we prepared nine peanut mixtures with matrices from different countries of origins and crop years and measured them by NMR spectroscopy.

MATERIALS AND METHODS

Reagents and chemicals

Deuterated solvents chloroform-*d* (99.8%) and methanol-d_4 (99.8%) were purchased from EURISOTOP (Saint-Aubin Cedex, France). Deuteriumoxide (99.9%), acetonitrile-d_3 (99.5%) and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (99.0%) were purchased from DEUTERO (Kastellaun, Germany). Sodium azide (99.5%), potassium phosphate monobasic anhydrous (>99%), potassium phosphate dibasic anhydrous (>98%), *trans*-4-hydroxy-L-proline (≥99%), *N*-methyl-*cis*-4-hydroxy-L-proline (≥98%) and sodium borohydride were purchased from SIGMA ALDRICH (Merck KGaA, Darmstadt, Germany). Formaldehyde (37%) was purchased from GRÜSSING GmbH (Filsum, Germany). Ultrapure water for reversed-phase chromatography was purified by a SARTORIUS arium® pro apparatus (Sartopore 0.2 µm, UV) and acetonitrile (HPLC grade) was purchased from VWR.

Chemical Analysis and Chromatography

Mass spectra were recorded on an AGILENT 6224 ESI-TOF instrument. The optical rotation was measured with a P8000 polarimeter from A. Krüss Optronic GmbH. For automated reversed-phase flash column chromatography a Sepacore® flash system from BÜCHI in combination with Flash RS 40 C_{18}ec column from MACHEREY-NAGEL was used. Method A: 0–10 min. H_2O (100%), flow rate 40 mL min^{-1},

fraction size 15 mL; Method B: 0–10 min. CH_3CN (0–50%), 10–12 (50–100%), 12–15 (100%), flow rate 40 mL min^{-1}, fraction size 10 mL or 5 mL. For preparative HPLC an Infinity system from AGILENT TECHNOLOGIES with a VP 250/10 Nucleodur C_{18} Pyramid column from MACHEREY-NAGEL was used. Method C: 0–10 min. CH_3CN (5%), 10–20 (5–95%), 20–25 (95%), flow rate 4 mL min^{-1}, fraction size: 1.33 mL.

Sample Preparation

The peanut samples (roasted, in shell) were purchased in local grocery stores in Hamburg (Germany) as detailed in the supplementary material table S1. The shell and skin were removed, 100 g of a sample were shock-frozen with liquid nitrogen and ground with 150 g dry ice using a Grindomix GM 300 knife mill equipped with a stainless-steel grinding container and a full metal knife (RETSCH, Haan, Germany). The powdered sample was freeze-dried for 48 hours and stored at -20 °C. Hazelnut samples were provided by producers and importers from a previous project.[22] Walnut and almond samples were purchased from different suppliers as detailed in the supplementary material table S1. Every nut sample was shock-frozen with liquid nitrogen and then the shell was removed. After the shock freezing was repeated, 100 g of the kernels were mixed with 150 g of dry ice and ground using a Grindomix GM 300 knife mill equipped with a stainless-steel grinding container and a full metal knife. The powdered sample was freeze-dried for 48 hours and stored at -20 °C.

Extractions

Extraction A: The series of mixtures were prepared by weighing the lyophilizate of peanut and a powdered sample of a food product (almond/hazelnut/walnut) with a total of 300 mg in a corresponding mixing ratio (Table 1). Then, two steel balls (Ø = 2 mm), 400 µL methanol-d_4, 500 µL chloroform-*d* and 600 µL deuterated phosphate buffer (50 mM) were added to the lyophilizate mixture and extracted in a

ball mill for three minutes at 3.1 m/s. Each sample was centrifuged at 14.000 rcf and 4 °C for ten minutes. Then, 100 µL of the supernatant was diluted with 600 µL phosphate buffer. Finally, 600 µL of the diluted extract were transferred to a 5 mm NMR tube. The extraction procedure was carried out as a triplicate for each sample.

Extraction B: For the methanol extract of the food products 300 mg lyophilizate was mixed with 1000 µL methanol-d_4 and two steel balls (Ø = 2 mm) were added. The mixture was extracted in a ball mill for three minutes at 3.1 m/s. It was centrifuged at 14.000 rcf and 4 °C for ten minutes. Finally, 600 µL of the extract were transferred to a 5 mm NMR tube.

Extraction C: For the methanol/acetonitrile extract of the food products 300 mg lyophilizate was mixed with 500 µL methanol-d_4 and 500 µL acetonitrile-d_3 and two steel balls (Ø = 2 mm) were added. The mixture was extracted in a ball mill for three minutes at 3.1 m/s and it was centrifuged at 14.000 rcf (4 °C) for ten minutes. Then, 350 µL of the supernatant was diluted with 350 µL methanol-d_4. Finally, 600 µL of the diluted extract were transferred to a 5 mm NMR tube.

Extraction D: For the acetonitrile extract of the food products 300 mg lyophilizate was mixed with 1000 µL acetonitrile-d_3 and two steel balls (Ø = 2 mm) were added. The mixture was extracted in a ball mill for three minutes at 3.1 m/s. It was centrifuged at 14.000 rcf and 4 °C for ten minutes. Finally, 600 µL of the extract were transferred to a 5 mm NMR tube.

Isolation of marker metabolite 1: A suspension of 6 g peanut lyophilizate in 60 mL methanol was stirred for one hour at room temperature. The solvent was removed under reduced pressure at 40 °C and the process was repeated. Then 60 mL water, 50 mL chloroform and 40 mL methanol were added to the

residue and the suspension was stirred for 90 minutes at room temperature. It was centrifuged at 14.000 rcf for 30 minutes. The aqueous phase was separated, the methanol was removed under reduced pressure at 40 °C and the water by lyophilization. The residue was dissolved in 15 mL water and filtered through Amicon® Ultra centrifugal filters (cut-off: 3 kDa) at 14.000 rcf for 15 minutes at room temperature. The filtrate was lyophilized, and the residue was separated by automated reversed-phase flash column chromatography using method A. The individual fractions were examined by ^{1}H NMR spectroscopy for the signal of the marker at 3.05 ppm and the relevant fractions were combined. The lyophilized residue of the combined fractions was analyzed a second time by automated reversed-phase flash column chromatography using method B. This procedure was repeated using method B with a smaller fraction size of 5 mL. After collection of the fractions showing the signal of the marker, 112 mg of lyophilized residue was separated by high-performance liquid chromatography using method C. The optical rotation of the fraction obtained showing the marker metabolite was $[\alpha]_D^{20}$= -7.1° (*c* 0.49, H_2O).

NMR acquisition

^{1}H NMR spectra were acquired on a Bruker Avance III HD 400 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 400.13 MHz. The noesygppr1d pulse sequence was used for acquisition of water suppressed ^{1}H NMR spectra applying the digitization mode baseopt. For each sample, two spectra with different numbers of scans (NS = 8, NS = 128) were recorded at 300 K with 65536 complex data points and a spectral width of 8417.5 Hz. The RG was set to 64 and the transmitter frequency offset to 1932.6 Hz.

^{1}H NMR spectra from polar extract of peanut for identification of the chemical marker were acquired on a Bruker Avance III HD 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 600.13 MHz. The zgesgp pulse sequence was used for acquisition of water suppressed ^{1}H NMR spectra of the fractionated polar peanut extract. The spectra were recorded at 298 K with NS = 128,

65536 complex data points and a spectral width of 9615.4 Hz. The RG was set to 64 and the transmitter frequency offset to 2821.8 Hz. For quantitative methods the relaxation delay D1 was set to 60 seconds and the transmitter frequency offset to 2898.63 Hz.

NMR data processing and analysis

The FIDs were Fourier transformed with a line broadening factor of 0.3 Hz. The spectra obtained were calibrated to the TMSP signal and processed by automatic phase and baseline correction with Topspin 3.5 (Bruker Biospins, Rheinstetten, Germany). For the series of mixtures, the signal-to-noise ratio (signal region: 3.0394 ppm to 3.0575 ppm, noise region: -1.000 ppm to -2.000 ppm) was determined automatically by using the implemented *sino* command. The obtained values were automatically extracted from the spectra data by using a Python script (supplementary material S9). The integral of the singlet at 3.05 ppm was determined with the same signal-region automatically for all spectra using the auto-integration modus of Topspin 3.5. The relative integrals were used to estimate of the biological variance of the marker metabolite by comparing them. The linear regressions were performed using the software Origin 2019 (OriginLab Corporation, Northampton, USA). The calculated mean of the admixture of peanut was plotted versus the mean signal-to-noise ratio.

Synthesis of *N*-methyl-*trans*-4-hydroxy-L-proline (1)

The method of Sondengam *et al.* was used to synthesize the metabolite *N*-methyl-*trans*-4-hydroxy-L-proline (1).[25] First, 208 mg (1.59 mmol) *trans*-4-hydroxy-L-proline were suspended in 4 mL methanol and 3 mL (0.03 mol) formaldehyde (37%) were added. The reaction mixture was refluxed for two hours. After cooling the solution to 0 °C, 308 mg (8.14 mmol) sodium borohydride was added and the mixture stirred for one hour at room temperature. The solvent was removed under reduced pressure and the residue was dissolved in 2 mL methanol and filtered. After removal of solvent under reduced pressure, the crude

product was separated by reversed-phase flush chromatography using method A. 15 mg were used for further purification with preparative HPLC using method C. The product was obtained after lyophilization of fraction no. 10 as a colorless solid. ^{1}H-NMR (deuterated phosphate buffer (50 mM, pH = 7), 600 MHz, 300 K): δ [ppm] = 4.66 – 4.62 (m, 1H, H-4), 4.20 (dd, $^{3}J_{H,H}$ = 7.5, 11.1 Hz, 1H, H-2), 3.96 (dd, 1H, $^{2}J_{H,H}$ = 13.0 Hz, $^{3}J_{H,H}$ = 4.8 Hz, 1H, H-5a/b), 3.20 (ddd, $^{2}J_{H,H}$ = 13.0 Hz, $^{4}J_{H,H}$ = 2.0, 2.0 Hz, 1H, H-5a/b), 3.05 (s, 3H, H-6), 2.49 (dddd, $^{2}J_{H,H}$ = 14.1 Hz, $^{3}J_{H,H}$ = 7.5 Hz, $^{4}J_{H,H}$ = 2.0, 2.0 Hz, 1H, H-3a/b), 2.26 (ddd, $^{2}J_{H,H}$ = 14.1 Hz, $^{3}J_{H,H}$ = 5.0, 11.1 Hz, 1H, H-3a/b). ^{13}C-NMR (deuterated phosphate buffer (50 mM, pH = 7), 151 MHz, 300 K): δ [ppm] = 172.9 (C-1), 70.0 (C-2), 69.4 (C-4), 62.6 (C-3), 43.1 (C-6), 38.2 (C-5). HRMS (ESI^{+}): For ([M+H]$^{+}$) calculated for $C_6H_{11}NO_3$: m/z = 146.0812, found: m/z = 146.0807. $[\alpha]_D^{20}$= -17.2° (*c* 0.50, H_2O).

RESULTS AND DISCUSSION

Peanuts are affected by food fraud because of their high availability and low price compared to other nuts, which is a challenging problem, especially if there is a chemical and visual similarity with the adulterated food. In order to detect peanut admixtures in powdered samples, an analytical marker, which must be individual for peanut extracts, is required. For this, four extractions of peanut sample and chemically similar raw materials (almond, hazelnut, walnut) with methanol-d_4, acetonitrile-d_3, acetonitrile-d_3/methanol-d_4 (1:1) and deuterated phosphate buffer/methanol-d_4/chloroform-*d* (2.5:2:3), were performed and analyzed by ^{1}H NMR spectroscopy. The spectra of peanut extracts were compared with all other spectra (supplementary material, Figure S1 – S3). Of these four extractions, only the ^{1}H NMR spectra obtained by the two polar solvents (A: deuterated phosphate buffer/methanol-d_4, B: methanol-d_4) show an individual marker signal (Extraction A: singlet at 3.05 ppm, extraction B: singlet at 3.02 ppm), which could indicate the admixture of peanut (Figure 1). The spectrum of peanut extract obtained by extraction A additionally shows three regions (δ = 3.17 – 3.19; 2.45 – 2.53; 2.20 – 2.27) in

which individual signals are observed, but with a lower intensity than the singlet at 3.05 ppm. Further, in the NMR spectra of hazelnut and almond extract B (methanol-d_4) signals with low intensity were detected in the spectral region of the singlet at 3.02 ppm (supplementary material, Figure S1). Since these signals can interfere and no such signals were observed in the spectra of extraction method A, the latter was selected for further analysis. Next, a total of nine different samples of the adulterated raw materials (hazelnut/almond/walnut; each from different countries of origin), were extracted and analyzed to verify they did not show any signal in the singlet area. It should be noted that there is a doublet of low intensity with a similar chemical shift of 3.04 ppm in the spectrum of aqueous walnut extract (Figure 1). In contrast, the spectra of hazelnut and almond extracts showed no signals in this region.

The stability of the potential marker was monitored at 3.05 ppm for one week at room temperature under observation of the singlet, with no changes in intensity (supplementary material, Figure S4). Figure S5 in the SI shows a spectrum of a peanut sample after roasting it for 6 hours at 180 °C. The peanuts turned mostly black under these conditions, but the metabolite, although partially degraded, was still detected in considerable amount. In addition, twelve roasted peanut samples of different origins (supplementary material, Figure S6) were purchased, measured and the presence of the chemical marker in all samples was confirmed. Therefore, the chemical marker belongs to the signal pattern of peanut extracts, regardless of the geographical origin. A biological variance of the marker signal was observed and further quantified. Ten individual kernels of three peanut samples, each from different countries (Egypt/South Africa/USA), were shock frozen and ground. Then, the powdered peanut kernels were extracted (Extraction A) and measured by ^{1}H NMR applying 128 scans. The relative integrals obtained were compared and the standard deviation was calculated for different intensities of the signal. Kernels from the USA exhibited the lowest standard deviation of 28%, a standard deviation of 37% was calculated for kernels from South Africa. Based on the total of 30 measured spectra of individual peanut kernels, an overall deviation of 46% was obtained for the marker singlet (supplementary material, Table S5). In

232 contrast to the variability of single kernels, the biological variance of the twelve peanut samples, prepared
233 from 100 g kernels each, showed a standard deviation of 14% (Table 2). The lowest absolute
234 concentration of the metabolite was calculated for peanut sample 12 with 5 mg mL^{-1} related to TMSP and
235 the highest was found in sample 11 with 9 mg mL^{-1}. Due to the biological variance, the use of the signal
236 of the marker for the accurate calculation of peanut admixtures is difficult. Nevertheless, it is suitable for
237 semi-quantitative and most important, for qualitative detection of peanut impurities. The first and most
238 important question in an incoming goods inspection will be whether or not the delivery of a product has
239 been adulterated by the addition of peanuts.
240
241 IDENTIFICATION OF THE PEANUT MARKER
242 The superimposition of individual signals makes the identification of metabolites in complex extraction
243 mixtures difficult, and 2D NMR experiments can also provide only limited information, since the
244 spectrum of the polar peanut extract is dominated by carbohydrate signals. The corresponding metabolite
245 with the singlet at 3.05 ppm in the NMR spectrum was identified after polar extraction and reversed-phase
246 column chromatography including fractionation to reduce the complexity of the spectrum. A ^{1}H NMR
247 spectrum (600 MHz) was measured of each fraction and all fractions showing the singlet were collected
248 and fractionated again. This was repeated three times and the metabolite was finally purified by HPLC in
249 one fraction (no. 10) with the metabolite as the main component (Figure 5). With the spectral data the
250 metabolite was identified as *N*-methyl-4-hydroxy-L-proline. This metabolite was first isolated and
251 described from peanut flour by Lee *et al.* in 1965.[26] However, it has not been characterized whether it is
252 the *cis*- or *trans*-4-hydroxy-L-proline derivative. The compound was confirmed by comparing the
253 chemical shifts of the signals with literature values (Haraguchi *et al.*[27]). The measured anticlockwise
254 rotation direction of $[\alpha]_D^{20} = -7.1°$ (*c* 0.49, H_2O) of the fraction no. 10 with the metabolite as a main
255 component indicates L-proline as the basic structure. In the ESI^+-MS spectrum the molecular peak was

observed for $[M+H]^+$ at m/z 146.0817. Additionally, two fragment ions were observed at m/z 100.0763 and m/z 82.0659, which had been described by Sciuto *et al*. for sequential losses of carboxyl and water.[28] As a reference, metabolite 1 was synthesized according to the method of Sondengam *et al*.[25] and used as an internal standard. An increase in intensity of the singlet and the associated signals in the fraction no. 10 was observed (Figure 5). Acquired *N*-methyl-*cis*-4-hydroxy-L-proline (2) was also used as an internal standard to exclude this structure. The optical rotation of metabolite 1 was measured as $[\alpha]_D^{20}$= -17.2° (*c* 0.50, H_2O) and an anticlockwise rotation direction was also indicated. This was compared with the rotation direction of the isolated product and literature and the absolute configuration was confirmed.[27,28] In this study it was shown that the metabolite in peanut is *N*-methyl-*trans*-4-hydroxy-L-proline (1). This metabolite has also been isolated from the poisonous plant *Ipomoea carnea*[27] and from leaf of five species of the leguminous tropical tree *Copaifera*.[29] In addition, metabolite 1 was found in red algae (*Chondria coerulescens*)[28] and higher plants of the species *Croton gubouga.*[30] Metabolite 1 was only described in pistachios as a nut-like food product.[31]

PEANUT ADULTERATION

Three series of mixtures (peanut/almond, peanut/hazelnut, peanut/walnut) were prepared by mixing the lyophilizates in specific mixing ratios (Table 1). The extraction (A) was carried out in triplicates and two spectra were acquired with different number of scans (NS = 8, NS = 128) for each individual sample. Both spectra were analyzed and compared regarding the detection limit using the signal-to-noise ratio (SNR) of the singlet. The signal region was set from 3.0394 ppm to 3.0575 ppm and the noise region from -1.000 ppm to -2.000 ppm. We used a Python script to extract the calculated SNR automatically from a series of spectra (supplementary material S9). The calculated mean values of the admixture of peanut were plotted versus the mean SNR values obtained. The coefficients of determination (R^2) in a range from 0.994 to 0.999 for both NS = 8 and NS = 128 show an adequate fit of the linear regressions. The

coefficients of determination, the approximate limit of detection and the calibration models obained are shown in table 3. Similar results of linear regression with comparable R^2 were obtained for the series of mixtures using the relative integral of the signal of the marker. For this purpose, the integrals in the spectra were determined automatically with the same signal region set from 3.0394 ppm to 3.0575 ppm. For hazelnut, both quantitation methods are compared graphically in figure 2. The graphical plots of almond and walnut mixture series are shown in supplementary figures S7 – S8 as well as the lists with the SNR values obtained with means and standard deviations for all series of mixtures in supplementary tables S2 – S4.

The change in the signal intensity of the marker in spectra of hazelnut extract with increasing peanut content is shown in comparison between NS = 8 and 128 as an example in figure 3. It is shown that an increase of the numbers of scans shows a slight improvement in qualitative detection of impurities with peanut. Using a lower number of scans resulted in very fast analysis times (~2 min), which allows a high sample throughput. Due to the natural variability of the signal of the marker, already discussed in the previous section, it is difficult to determine an exact value for the limit of detection (LOD). With regard to the sample used for the hazelnut/peanut (peanut 2) and almond/peanut (peanut 1) calibration model, the signal of the marker showed an $SNR \geq 3$ in the spectra (NS = 8) with an admixture of 4% peanut. However, this method cannot be applied e.g. in the walnut/peanut mixture series since a certain signal-to-noise ratio is already present in the blank sample due to the doublet which is present in the walnut spectra with a similar chemical shift of 3.04 ppm. Nevertheless, with the calibration models obtained semi-quantitative predictions of peanut adulteration in powdered matrices can be made.

QUANTITATION OF PEANUT SAMPLES

For quantitation of unknown mixtures with the models obtained from linear regression, samples with peanut content from 1.5% to 27% were prepared and extracted (method A) in triplicates. The samples

used for these mixtures, both peanut and adulterated food, were from different countries and harvest years. The focus of the evaluation was on the errors that occurred in the calculations due to the natural variance of the marker. The true value of peanut content was calculated by using the mean value of the triplicate. The linear calibration models (Table 3) of the three series of mixtures resulted from evaluation by SNR were used for calculating the peanut content using mean of the obtained SNR values from triplicates. In table 4 the results of quantitation with the linear regression model (almond) are shown. As an example, the true peanut content of the last sample was 27% and the calculated impurity was 16% peanut by means of NS = 8. The result shows the highest deviation of 40% and in this case, the peanut sample 1 (declared origin: Egypt) was used for calibration and the sample (peanut 12) used in this mixture has a significantly lower concentration of the marker metabolite (Figure 4). Peanut sample 12 was conspicuous in the set of all 12 peanut samples because the concentration of the marker was the lowest, which is highlighted in red in the box plot in Figure 4. This affects the calculation, as the calculated value is also significantly lower than the true value. The calculated value depends on the peanut sample used as adulterant and the similarity of the concentration of the chemical marker in relation to the sample used for calibration. This is a difficult problem for accurate quantitation as the content of the marker metabolite in a peanut sample used as adulterant is most likely unknown. If the concentration of the metabolite in the peanut sample used in calibration is similar to the concentration of the added sample (e.g. peanut 10), then a small deviation in calculation is obtained, resulting in an accurate calculation of the peanut content. This can be seen in the example of using peanut 10 as adulterant, where the deviation is only 1%. On average, for nine peanut adulterated almond samples a deviation of 15 ─ 16% was calculated for both number of scans (NS = 8, 128). Similar results were obtained in quantitation of walnut and hazelnut samples (supplementary table S6 and S7). When adulterating walnut with peanut, the calculated peanut content deviates on average by 23 ─ 26% from the true value whereas in hazelnut samples the average deviation from true peanut content was 20 ─ 29%. The deviations reflect the error resulting from the different

content of the marker metabolite in the peanut samples. Nevertheless, the semi-quantitative calculation provides a good method for estimating the peanut content. An important advantage is that variations of adulterated food, e.g. geographical origin or varieties, do not affect on the calculation as long as no other signals occur in the range of the peanut marker signal at 3.05 ppm. In addition, the signal of *N*-methyl-*trans*-4-hydroxy-L-proline (1) provides a reliable qualitative indication of peanut in unknown food samples.

If peanut mixed with foods that are visually and chemically similar, this poses a challenging problem for quality control and therefore it is important to develop fast methods for detection of peanut adulteration. The detection of peanut adulteration in various food products (almond, hazelnut, walnut) was performed using NMR spectroscopy in combination with an individual marker signal (singlet at 3.05 ppm). The selected signal of the marker is universally applicable for the detection of economic relevant adulteration with peanuts in visually similar raw materials as presented in this study. By extraction from powdered peanut in combination with reversed-phase chromatography and HPLC, the signal used as a marker was assigned to the metabolite *N*-methyl-*trans*-4-hydroxy-L-proline (1). Identification was based on the comparison with the synthesized product 1 and was confirmed by MS and NMR data. This metabolite has high potential to be used in routine analysis with fast acquisition times for the identification of adulterations with peanuts. A major advantage of using this marker is the belonging to the metabolic profile of peanuts, as the metabolite was already discovered in samples from 1965 and is also present in the samples used in this study that were acquired in 2019.[26] With a spectral acquisition time of two minutes applying 8 number of scans, an approximate limit of detection of 4% adulteration of peanuts was reached. The LOD can easily be reduced by extending the acquisition time. However, it should be noted that the method is not sensitive enough to detect peanut allergens, but economic relevant adulterations, such as those shown in the operation OPSON VI (8%) can be detected.[24] The semi-quantitative method is influenced by the biological variance of the chemical marker, but is not dependent on the adulterated raw

352 material. In the context of food fraud, NMR spectroscopy thus represents a viable screening method for
353 detection of peanut adulteration.

TABLES

Table 1: Ratios of the three series of mixtures prepared from two peanut samples (peanut 1/peanut 2). The geographical origins of the food products were labelled as: Egypt/Italy (peanut/almond), Egypt/France (peanut/walnut) and USA/France (peanut/hazelnut).

peanut [%]	food product [%]	peanut [%]	food product [%]
100	0	8	92
90	10	6	94
80	20	4	96
70	30	2	98
60	40	1	99
50	50	0.8	99.2
40	60	0.6	99.4
30	70	0.4	99.6
20	80	0	100
10	90		

Table 2: Relative integral of the singlet (3.05 ppm) in the polar extract of twelve different peanut samples. The biological variance of the chemical marker was observed calculating the standard deviation of 14%.

peanut sample	declared origin	relative integral
1	Egypt	1.36
2	USA	1.25
3	Israel	1.25
4	South Africa	1.31
5	USA	1.31
6	Egypt	1.09
7	Egypt	1.18
8	USA	1.23
9	USA	1.29
10	USA	1.28
11	Egypt	1.68
12	Egypt	0.89
	mean	1.26
	standard deviation [%]	14

Table 3: Approximate limit of detection of the series of mixtures and calibration model obtained. The coefficients of determination of linear regression for both evaluations show similar results. With a higher number of scans of 128 a lower LOD can be obtained due to higher signal-to-noise ratio.

peanut mixture	NS	R^2 (SNR/integral)	LOD	linear calibration model (SNR)
walnut	8	0.994/0.998	>4%	$y = 0.77721 + 1.16327 \cdot x$
	128	0.997/0.999	>2%	$y = 4.92591 + 4.69144 \cdot x$
almond	8	0.996/0.998	>4%	$y = 0.31802 + 1.16597 \cdot x$
	128	0.995/0.998	>2%	$y = 3.36174 + 4.66916 \cdot x$
hazelnut	8	0.997/0.998	>4%	$y = 0.99714 + 0.98465 \cdot x$
	128	0.997/0.998	>2%	$y = 5.23451 + 3.93275 \cdot x$

Table 4: Results of the quantitation of almond adulteration with peanut samples from various countries. Almond samples were of different geographical origin and harvest years. Due to the biological variance of the chemical marker, the calculated values deviate on average by 15 ─ 16% from the true value.

almond sample[1]	NS	SNR[2]	calculated value [%][3]	peanut [%][2]	deviation [%]	peanut sample[1]
1	8	2.46	1.84	1.51	21.57	4
	128	9.92	1.40		7.05	
9	8	3.51	2.74	3.00	8.83	5
	128	15.94	2.69		10.32	
2	8	4.83	3.87	5.12	24.50	6
	128	21.41	3.87		24.56	
7	8	8.56	7.07	7.05	0.23	8
	128	33.01	6.35		9.91	
8	8	11.59	9.67	9.02	7.14	7
	128	45.50	9.02		0.02	
3	8	15.35	12.89	13.02	0.94	9
	128	63.10	12.79		1.70	
4	8	20.38	17.21	17.98	1.32	10
	128	81.76	16.79		1.14	
5	8	38.02	32.34	23.02	40.48	11
	128	154.73	32.42		40.85	
6	8	19.12	16.13	27.05	40.38	12
	128	79.28	16.26		39.89	

[1]specifications of samples as detailed in the supplementary material table S1; [2]mean of triplicates; [3]calculation was performed using the calibration model in table 3 (almond).

FIGURES

Figure 1

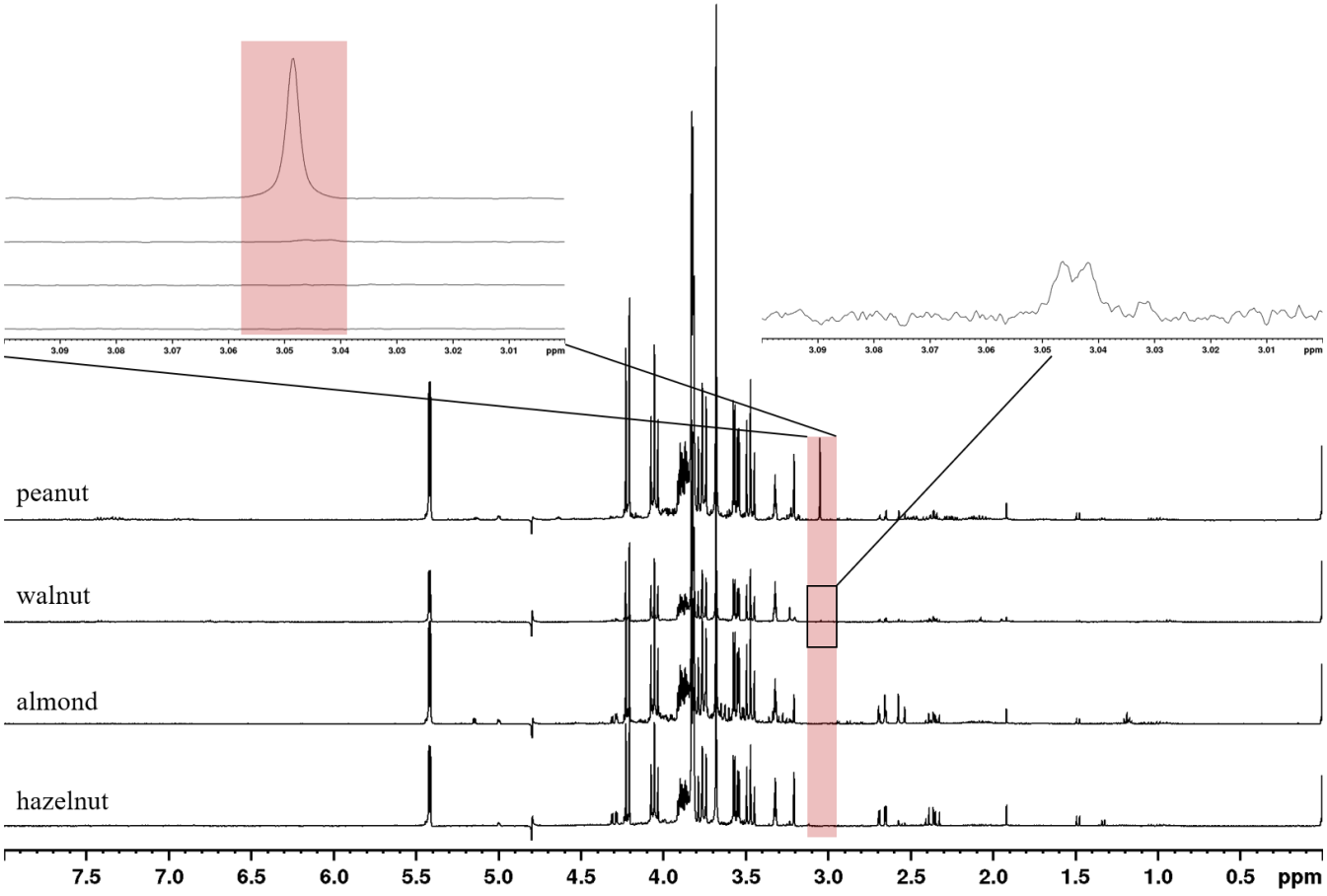


Figure 2

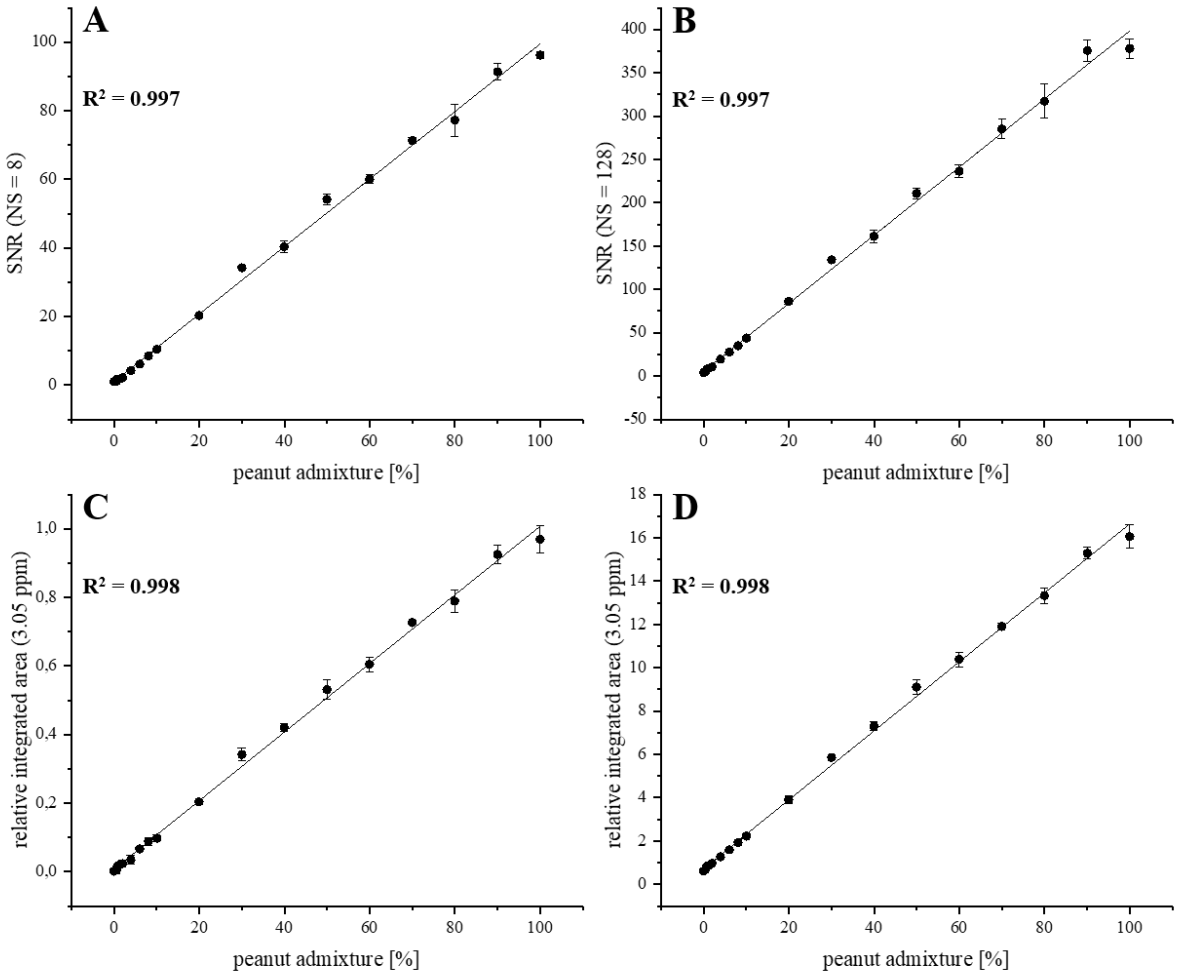

A
R² = 0.997
SNR (NS = 8)
peanut admixture [%]
B
R² = 0.997
SNR (NS = 128)
peanut admixture [%]
C
R² = 0.998
relative integrated area (3.05 ppm)
peanut admixture [%]
D
R² = 0.998
relative integrated area (3.05 ppm)
peanut admixture [%]

Figure 3

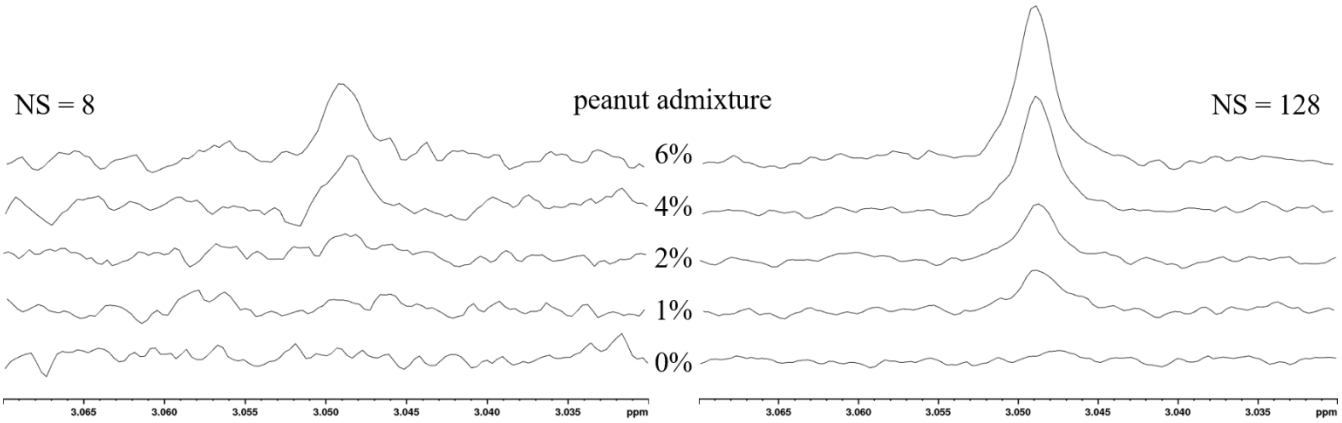

NS = 8
peanut admixture
NS = 128
6%
4%
2%
1%
0%
3.065
3.060
3.055
3.050
3.045
3.040
3.035
ppm

Figure 4

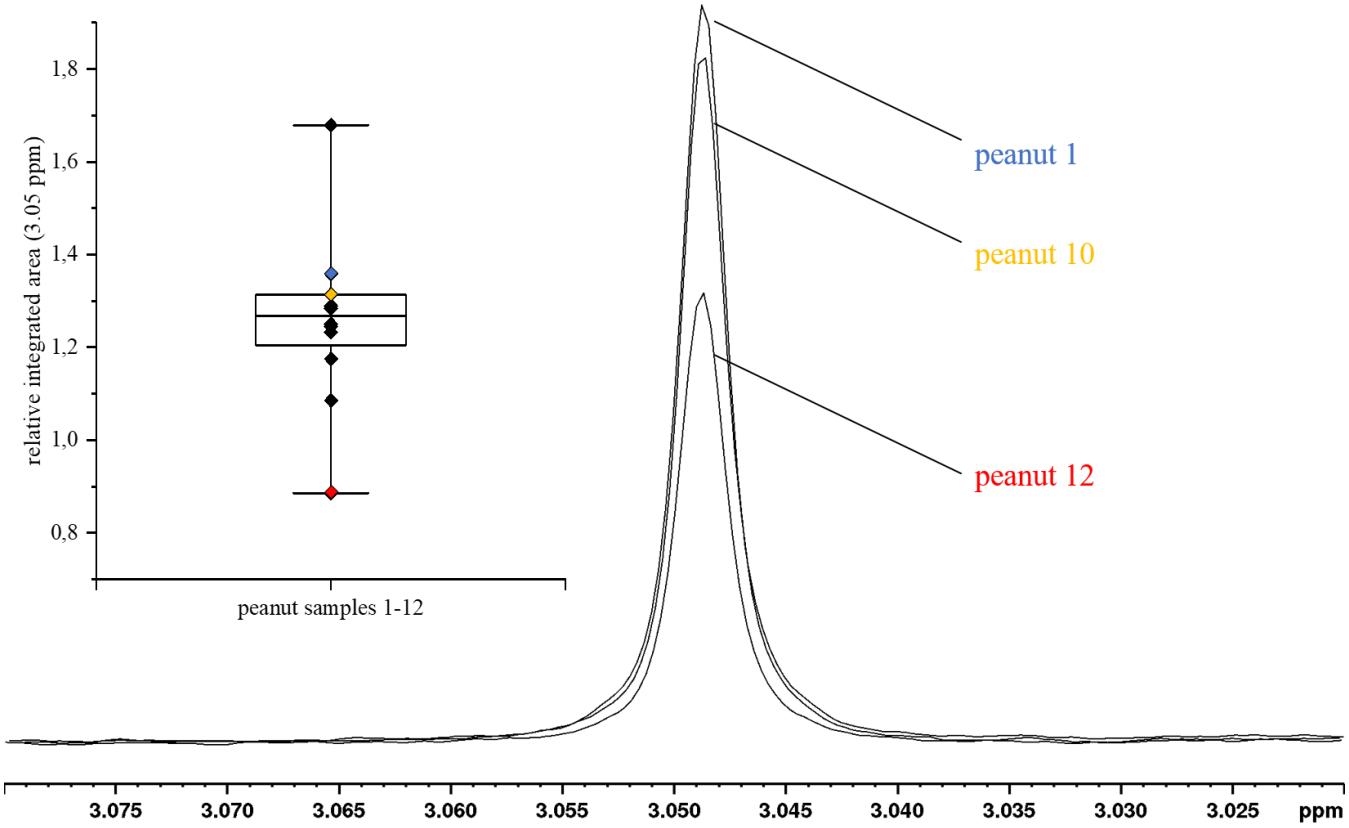
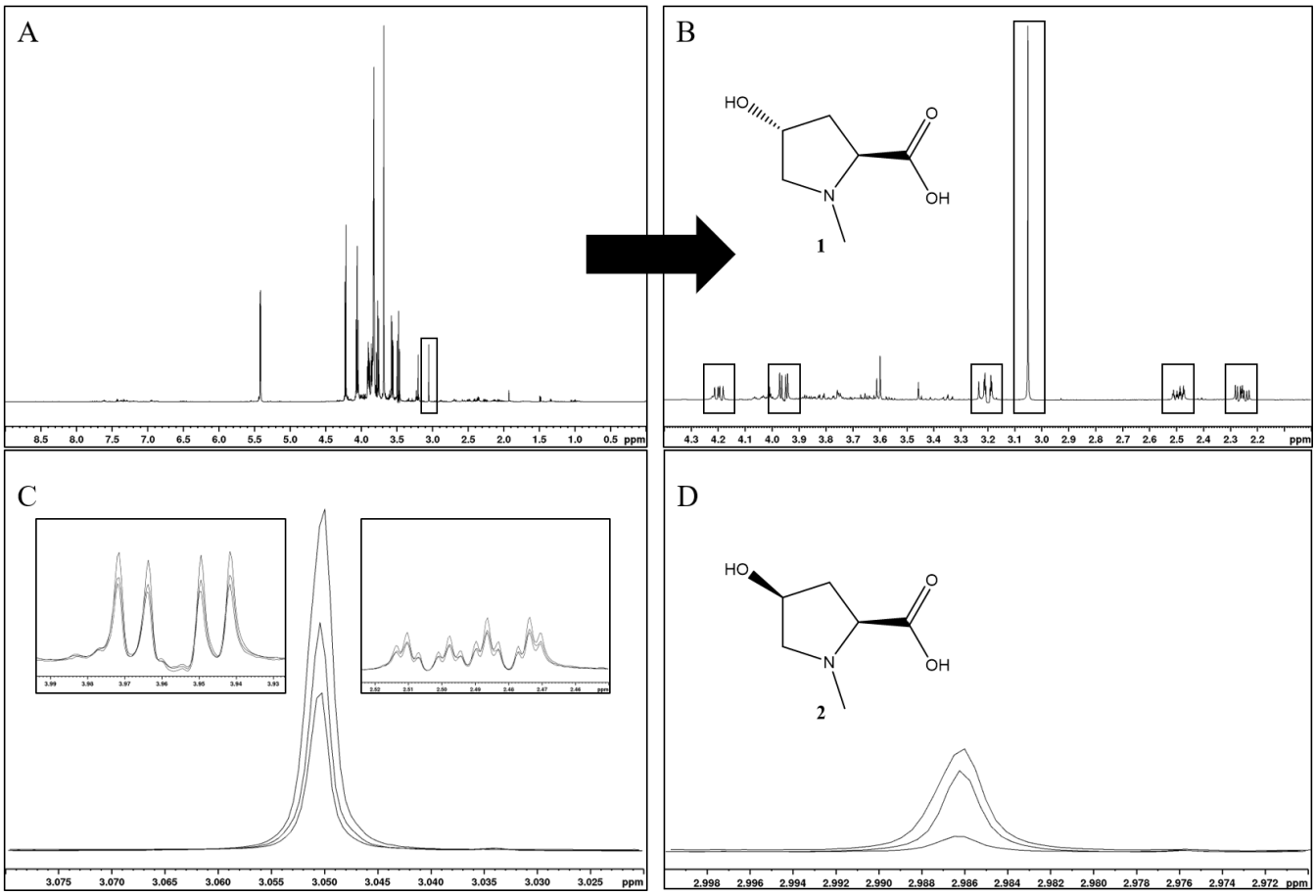

relative integrated area (3.05 ppm)
1,8
1,6
1,4
1,2
1,0
0,8
peanut samples 1-12
peanut 1
peanut 10
peanut 12
3.075
3.070
3.065
3.060
3.055
3.050
3.045
3.040
3.035
3.030
3.025
ppm

Figure 5



ASSOCIATED CONTENT

Supporting Information

The following supporting information is available free of charge at ACS website http://pubs.acs.org:

Table S1: Information for nut samples used in this study including supplier, harvest year, variety and declared origin.

Table S2: List of peanut additives with mean and standard deviation used in the almond series of mixtures. The signal-to-noise ratio (NS = 8, NS = 128) obtained with mean and standard deviation for the linear regression is shown.

Table S3: List of peanut additives with mean and standard deviation used in the walnut series of mixtures. The signal-to-noise ratio (NS = 8, NS = 128) obtained with mean and standard deviation for the linear regression is shown.

Table S4: List of peanut additives with mean and standard deviation used in the hazelnut series of mixtures. The signal-to-noise ratio (NS = 8, NS = 128) obtained with mean and standard deviation for the linear regression is shown.

Table S5: Relative integrals of singlet at 3.05 ppm of individual peanut kernels with declared origin and calculation of mean and standard deviation.

Table S6: Results of the quantitation of hazelnut adulteration with peanut samples from various countries. Hazelnut samples were of different geographical origin and harvest years. Due to the biological variance of the chemical marker, the calculated values deviate on average by 20 ─ 29% from the true value.

Table S7: Results of the quantitation of walnut adulteration with peanut samples from various countries. Walnut samples were of different geographical origin and harvest years. Due to the biological variance of the chemical marker, the calculated values deviate on average by 23 ─ 26% from the true value.

Figure S1: ^{1}H NMR spectra (400 MHz, NS = 128) of the methanol-d_4 extract of food products. The area of the potential marker signal around 3.05 ppm is shown expanded. The extraction method was not used for mixture analysis, because of other signals appearing in the same region as the marker singlet.

Figure S2: ^{1}H NMR spectra (400 MHz, NS = 128) of the acetonitrile-d_3 extract of food products.

Figure S3: ^{1}H NMR spectra (400 MHz, NS = 128) of the methanol-d_4/acetonitrile-d_3 (1:1) extract of food products.

Figure S4: ^{1}H NMR spectra (400 MHz, NS = 128) of the stability measurement of a peanut extract measured on the day of extraction (day 0), on the following two days (day 1, day 2) and after one week (day 7) are shown. The area of the marker signal around 3.05 ppm is shown expanded. The signal of the marker is stable, because no changes in signal intensities were observed.

Figure S5: ^{1}H NMR spectra (400 MHz, NS = 128) of ten kernels of purchased peanut sample 8 (roasted), which were shock-frozen and mortared before polar extraction. The marker metabolite was still present after roasting ten kernels of the same sample for six hours at 180 °C.

Figure S6: ^{1}H NMR spectra (400 MHz, NS = 128) of twelve peanut extracts using extraction method A. The presence of the chemical marker in peanut samples was confirmed as the singlet at 3.05 ppm was detected in all measured spectra.

Figure S7: Graphical plots of the calculated peanut quantity in mixture samples with almond versus the obtained signal-to-noise ratio of spectra measured with NS = 8 (A) and NS = 128 (B). The evaluation was also performed via the relative integrated area of the marker singlet at 3.05 ppm. The linear regression of the relative integrated area of the spectra obtained with NS = 8 (C) and NS = 128 (D) is shown.

Figure S8: Graphical plots of the calculated peanut quantity in mixture samples with walnut versus the obtained signal-to-noise ratio of spectra measured with NS = 8 (A) and NS = 128 (B). The evaluation was also performed via the relative integrated area of the marker singlet at 3.05 ppm. The linear regression of the relative integrated area of the spectra obtained with NS = 8 (C) and NS = 128 (D) is shown.

S9: Python script: The script is for extracting automatically signal-to-noise (*sino*) values obtained with TopSpin 3.5 (Bruker cooperation) from the raw of NMR spectra into a text file.

AUTHOR INFORMATION

Corresponding Author

* Corresponding author: Dr. Thomas Hackl, Institute of Organic Chemistry, University of Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany Tel.: +49-40-428382804 E-Mail: Thomas.Hackl@chemie.uni-hamburg.de

Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

NOTE

The authors declare no competing financial interest.

FUNDING

The project is supported by funds of the Federal Ministry of Food and Agriculture (BMEL; "Food Profiling", funding code: 2816500914) by a decision of the German Bundestag (parliament). Project support is provided by the Federal Institute for Agriculture and Food (BLE) within the scope of the program for promoting innovation. The consortium is headed by the HAMBURG SCHOOL OF FOOD SCIENCE. The focus lies on developments in the area of instrumental analysis for the authentication of foodstuffs.

ACKNOWLEDGMENT

The authors are very grateful to René Bachmann and Maike Arndt providing us with authentic nut samples. The authors thank Vera Priegnitz and Claudia Wontorra for their support in sample measurement.

ABBREVIATIONS

AF, Afghanistan; AU, Australia; ELISA, enzyme-linked immunosorbent assay; EG, Egypt; ES, Spain; ESI, electrospray ionization; FID, free induction decay; IT, Italy; LOD, limit of detection; NIR, near-

427 infrared reflectance spectroscopy; NS, number of scans; 16-OMC, 16-*O*-methylcafestol; PCR,
428 polymerase chain reaction; RG, receiver gain; SNR, signal-to-noise ratio; TMSP, trimethylsilylpropionic
429 acid; US, United States of America; ZA, South Africa.

REFERENCES

1. Ghosh S.; Mishra P.; Mohamad S. N. H.; de Santos R. M.; Iglesias B. D.; Elorza P. B. Discrimination of peanuts from bulk cereals and nuts by near infrared reflectance spectroscopy. *Biosyst Eng*. **2016**, *151*, 178─186.

2. Klockmann S.; Reiner E.; Cain N.; Fischer M. Food Targeting: Geographical Origin Determination of Hazelnuts (Corylus avellana) by LC-QqQ-MS/MS-Based Targeted Metabolomics Application. *J. Agric. Food Chem*. **2017**, *65*(7), 1456─1466.

3. Esteki M.; Farajmand B.; Kolahderazi Y.; Simal-Gandara J. Chromatographic Fingerprinting with Multivariate Data Analysis for Detection and Quantification of Apricot Kernel in Almond Powder. *Food Anal. Methods* **2017**, *10*(10), 3312─3320.

4. González M.; Gallego M.; Valcárcel M. Determination of natural and synthetic colorants in prescreened dairy samples using liquid chromatography-diode array detection. *Anal. Chem*. **2003**, *75*(3), 685─693.

5. Ding X.; Ni Y.; Kokot S. NIR spectroscopy and chemometrics for the discrimination of pure, powdered, purple sweet potatoes and their samples adulterated with the white sweet potato flour. *Chemom. Intell. Lab. Syst*. **2015**, *144*, 17─23.

6. Yang Y., Ferro M. D.; Cavaco I.; Liang Y. Detection and Identification of Extra Virgin Olive Oil Adulteration by GC-MS Combined with Chemometrics. *J. Agric. Food Chem*. **2013**, *61*(15), 3693─3702.

7. Fang G.; Goh J. Y.; Tay M.; Lau H. F.; Li S. F. Y. Characterization of oils and fats by 1H NMR and GC/MS fingerprinting: Classification, prediction and detection of adulteration. *Food Chem*. **2013**, *138*(2-3), 1461─1469.

8. Mannina L.; D'Imperio M.; Capitani D.; Rezzi S.; Guillou C.; Mavromoustakos T.; Vilchez M. D. M.; Fernández A. H.; Thomas F.; Aparicio R. ^{1}H NMR-based protocol for the detection of adulterations of refined olive oil with refined hazelnut oil. *J. Agric. Food Chem*. **2009**, *57*(24), 11550─11556.

9. Hu Y.; Wang S.; Wang S.; Lu X. Application of nuclear magnetic resonance spectroscopy in food adulteration determination: The example of Sudan dye i in paprika powder. *Sci. Rep*. **2017**, *7*(1), 1─9.

10. Monakhova Y. B.; Ruge W.; Kuballa T.; Ilse M.; Winkelmann O.; Diehl B.; Thomas F.; Lachenmeier D. W. Rapid approach to identify the presence of Arabica and Robusta species in coffee using ^{1}H NMR spectroscopy. *Food Chem*. **2015**, *182*, 178─184.

11. Gunning Y.; Defernez M.; Watson A. D.; Beadman N.; Colquhoun I. J.; Le Gall G.; Philo M.; Garwood H., Williamson D.; Davis A. P.; Kemsley E. K. 16-O-methylcafestol is present in ground roast Arabica coffees: Implications for authenticity testing. *Food Chem*. **2018**, *248*, 52─60.

12. Schievano E.; Finotello C.; De Angelis E.; Mammi S.; Navarini L. Rapid Authentication of Coffee Blends and Quantification of 16-O-methylcafestol in Roasted Coffee Beans by Nuclear Magnetic Resonance. *J. Agric. Food Chem*. **2014**, *62*, 12309─12314.

13. Parker T.; Limer E.; Watson A. D.; Defernez M.; Williamson D.; Kemsley E. K. 60 MHz ^{1}H NMR spectroscopy for the analysis of edible oils. *Trends Anal. Chem*. **2014**, *57*, 147─158.

14. Bachmann R.; Shakiba N.; Fischer M.; Hackl T. Assessment of Mixtures by Spectral Superposition. An Approach in the Field of Metabolomics. *J. Proteome Res*. **2019**, *18*(6), 2458─2466.

15. Esteki M.; Vander Heyden Y.; Farajmand B.; Kolahderazi Y. Qualitative and Quantitative Analysis of Peanut Adulteration in Almond Powder Samples Using Multi-Elemental Fingerprinting Combined with Multivariate Data Analysis Methods. *Food Control* **2017**, *82*, 31─41.

16. Scaravelli E.; Brohée M.; Marchelli R.; van Hengel A. J. Development of three real-time PCR assays to detect peanut allergen residue in processed food products. *Eur. Food Res. Technol*. **2008**, *227*(3), 857─869.

17. Stephan O.; Vieths S. Development of a real-time PCR and a sandwich ELISA for detection of potentially allergenic trace amounts of peanut (Arachis hypogaea) in processed foods. *J. Agric. Food Chem*. **2004**, *52*(12), 3754─3760.

18. Hefle S. L.; Bush R. K.; Yunginger J. W.; Chu F. S. A Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for the Quantitation of Selected Peanut Proteins in Foods. *J. Food Prot*. **1994**, *57*(5), 419─423.

19. Mills E. N. C.; Potts A.; Plumb G. W.; Lambert N.; Morgan M. R. A. Development of a rapid dipstick immunoassay for the detection of peanut contamination of food. *Food Agric. Immunol*. **1997**, *9*(1), 37─50.

20. Planque M.; Arnould T.; Delahaut P.; Renard P.; Dieu M.; Gillard N. Development of a strategy for the quantification of food allergens in several food products by mass spectrometry in a routine laboratory. *Food Chem*. **2019**, *274*, 35─45.

21. Pilolli R.; De Angelis E.; Monaci L. In house validation of a high resolution mass spectrometry Orbitrap-based method for multiple allergen detection in a processed model food. *Anal. Bioanal. Chem*. **2018**, *410*, 5653─5662.

22. Bachmann R.; Klockmann S.; Haerdter J.; Fischer M.; Hackl T. ^{1}H NMR Spectroscopy for Determination of the Geographical Origin of Hazelnuts. *J. Agric. Food Chem*. **2018**, *66*(44), 11873─11879.

23. Wang H.; Yuan F.; Wu Y.; Yang H.; Xu B.; Liu Z.; Chen Y. Detection of allergen walnut component in food by an improved real-time PCR method. *J. Food Prot*. **2009**, *72*(11), 2433─2435.

24. Europol/INTERPOL. Operation OPSON VI - Targeting Counterfeit and Substandard Foodstuff and Beverage: https://ec.europa.eu/food/sites/food/files/safety/docs/official-controls-food-fraud_opson-vi-report.pdf. (accessed 05.09.2019).

25. Sondengam B. L.; Hémo J. H.; Charles G. Sur une nouvelle methode de methylation des amines primaires et secondaires. *Tetrahedron Lett*. **1973**, *3*, 261─263.

26. Lee L. S.; Morris N. J.; Frampton V. L. Peanut Flour Constituents - Cyclic Imino Acid Derivative from Peanut Flour. *J. Agric. Food Chem*. **1965**, *13*(4), 309─311.

27. Haraguchi M.; Gorniak S.L.; Ikeda K.; Minami Y.; Kato A.; Watson A. A.; Nash R. J.; Molyneux R. J.; Asano N. Alkaloidal components in the poisonous plant, *Ipomoea carnea* (Convolvulaceae). *J. Agric. Food Chem*. **2003**, *51*(17), 4995─5000.

28. Sciuto S.; Chillemi R.; Piattelli M.; Impellizzeri G. The identification of 4-hydroxy-*N*-methylproline in the red alga *Chondria Coerulescens* - spectral information. *Phytochemistry* **1983**, *22*(10), 2311─2312.

29. Figliuolo R.; Naylor S.; Wang J.; Langenheim J. H. Unusual nonprotein imino acid and its relationship to phenolic and nitrogenous compounds in *Copaifera*. *Phytochemistry* **1987**, *26*(12), 3255─3259.

30. Goodson J. A.; Clewer H. W. B. Examination of the Bark of Croton gubouga. Isolation of 4-Hydroxyhygric Acid. *J. Chem. Soc. Trans*. **1919**, *115*, 923─933.

31. Sciubba F.; Capuani G.; Di Cocco M. E.; Avanzato D.; Delfini M. Nuclear magnetic resonance analysis of water soluble metabolites allows the geographic discrimination of pistachios (*Pistacia vera*). *Food Res. Int*. **2014**, *62*, 66─73.

521 FIGURE CAPTIONS

522 Figure 1: ^{1}H NMR spectra (400 MHz, NS = 128) of polar extraction of food products showing the
523 individual marker singlet at 3.05 ppm only in the peanut spectrum. In the spectrum of walnut extract there
524 is a doublet of lower intensity at 3.04 ppm (magnification of factor 10).

525 Figure 2: Graphical plots of the calculated peanut quantity in mixture samples (hazelnut) versus the
526 obtained signal-to-noise ratio of spectra measured with NS = 8 (A) and NS = 128 (B). The evaluation was
527 also performed via the integrated area of the marker singlet at 3.05 ppm. The linear regression plots of the
528 integrated area of the spectra obtained with NS = 8 (C) and NS = 128 (D).

529 Figure 3: ^{1}H NMR spectra (400 MHz) recorded with NS = 8 and NS = 128 of polar extraction of hazelnut
530 adulterated with peanut (0 – 6%) showing the marker singlet at 3.05 ppm. An approximate limit of
531 detection of 2 ─ 4% for the hazelnut series of mixture was observed.

532 Figure 4: ^{1}H NMR spectra (400 MHz, NS = 128) of polar extraction of peanut samples showing the marker
533 singlet at 3.05 ppm. Peanut sample 1 was used for calibration of the almond and walnut series of mixtures.
534 In examination of prepared almond samples adulterated with peanut, a high deviation (40%) of the true
535 value from calculated peanut content was obtained using peanut sample 12 as adulterant. Due to similar
536 intensity of the signal and concentration of the chemical marker a low deviation (1%) was calculated for
537 mixtures with peanut sample 10. The box plot shows the minimum and maximum values of the relative
538 integrals of the signal of the marker for twelve peanut samples used in this study. The three values of the
539 integrals of the peanut samples 1, 10 und 12 are highlighted in color.

540 Figure 5: (A) ^{1}H NMR spectrum (600 MHz, NS = 128) of polar peanut extract. (B) The metabolite
541 (2*S*,4*R*)-*N*-methyl-4-hydroxy-L-proline (1) was obtained with few impurities by three times reversed-
542 phase chromatography followed by preparative HPLC. (C) The presence of the metabolite 1 was
543 confirmed by using the synthesized metabolite *N*-methyl-*trans*-4-hydroxy-L-proline (1) as internal

544 standard. An increase of the marker singlet at 3.05 ppm was observed. (D) *Spike-in* experiment using the

545 purchased metabolite *N*-methyl-*cis*-4-hydroxy-L-proline (2) shows an increase of singlet at 2.99 ppm.

TABLE OF CONTENT GRAPHIC