Differentiation of Truffle Species (*Tuber* spp.) by ^{1}H NMR Spectroscopy and support vector machine

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ABSTRACT: The price of different truffle types varies according to their culinary value, sometimes by more than a factor of ten. Non-professionals can hardly distinguish the species within the white or black truffles by eye, which makes the possibility of food fraud very easy. Therefore, the identification of different truffle species (*Tuber* spp.) is an analytical task that could be solved in this study. The polar extract from a total of 80 truffle samples were analyzed by ^{1}H NMR spectroscopy in combination with chemometric methods covering five commercially relevant species. All classification models were validated with nested cross-validation. The two very similar looking and closely related black representatives *Tuber melanosporum* and *Tuber indicum* could be classified 100% correctly in direct comparison. The most expensive truffle *Tuber magnatum* could be distinguished 100% from the other relevant white truffle *Tuber borchii*. Furthermore, signals for a potential *Tuber borchii*, and a potential *Tuber melanosporum* marker for targeted approaches could be detected and the corresponding molecules were identified as betaine and ribonate. A model covering all five truffle species *Tuber aestivum*, *Tuber borchii*, *Tuber indicum*, *Tuber magnatum* and *Tuber melanosporum* was able to correctly discriminate between all species.

KEYWORDS: *truffle, Tuber spp., metabolomics, NMR, species authentication, food profiling, support vector machine*

1. INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy has become increasingly important in the field of metabolomics over the last two decades.[1] Metabolomics is mainly focused on the identification of low molecular weight molecules (<1500 Da) of a biological system. Metabolites are present in a wide dynamic concentration range and high chemical diversity.[2-4] For this reason, mass spectrometry and NMR are suited for their analysis because they are capable of analyzing a large number of metabolites in just a single measurement.[5] NMR is non-destructive and does not require pre-selection of analytical parameters, such as ionization conditions or chromatographic settings in mass spectrometry, where NMR has significantly lower sensitivity compared to mass spectrometry methods.[6,7] The main advantages of NMR over mass spectrometry are the good repeatability and reproducibility of the measurements and the proportionality of the signal integrals to the concentrations of the molecules measured.[8] NMR is therefore a powerful tool to determine the metabolism of different species or of the same species from different origins on the basis of concentration differences of the molecules contained. For example, it is possible to determine the geographical origin of several different foodstuffs, like roasted coffee,[9] wine,[10,11] or olive oil,[12,13] relatively accurately using NMR.[14]

Truffles are edible fungi in the genus *Tuber*, of the class Ascomycetes (Division Mycota), which grow in ectomycorrhizal symbiosis with several tree and shrub species below the ground.[15,16] Many volatile compounds are produced in the fruiting bodies that serve as attractants for insects and mammals.[16] The unique olfactory aromas and the special taste of truffles, are the reason for their culinary value and make some truffle species the most expensive edible fungi in the world and even one of the most valuable foods.[17,18] Since truffles are difficult to cultivate, they require time-consuming seeking, which is also reflected in the price of the fungi.[19] At the same time, prices for different species can vary widely. The white truffle species *Tuber magnatum*, named by Vittorio Picco,[20] also known as Piemont or Alba truffle, mainly distributed in Italy, produces a distinctive aroma and is the most valuable among the truffles.[21] Moreover, they are also found in France, Switzerland, and Eastern Europe.[21,22] Market prices range from 1000-5000 €/kg, depending on the origin, year of harvest and quality.[23] *Tuber borchii*, another white truffle species with prices between 105 and 305 €/kg[23] and thus a lower economic value,

is morphologically and biochemically similar to *Tuber magnatum* and is therefore readily used to adulterate *Tuber magnatum*.[24,25] The natural flavor of *Tuber magnatum* can also be mimicked in processed foods by the addition of bis(methylthio)methane.[26] It is also possible that white truffles, like *Tuber borchii*, grow on trees that were originally in symbiosis with *Tuber magnatum* and are therefore more difficult to distinguish.[27] (As *Tuber albidum* Picco and *Tuber borchii* Vittad. are synonyms for the same species, the name *Tuber borchii* is used in this work covering *Tuber albidum* Picco and *Tuber borchii* Vittad.[20]). A further example is the precious black truffle *Tuber melanosporum*, named by Carlo Vittadini,[28] that achieves market prices of 950-4000 €/kg due to its special taste and smell.[18,29] The Périgord truffle, as *Tuber melanosporum* is also called, grows mainly in France, Spain and Italy.[22] Since *Tuber melanosporum* as an originally European fungus can also be cultivated with some effort, it is now farmed in Australia as well.[30] Morphologically very similar to *Tuber melanosporum* is its Asian relative *Tuber indicum*.[31] (*Tuber indicum*, named by Mordecai Cubitt Cooke and George Edward Massee,[32,33] *Tuber himalayense* and *Tuber sinense* were summarized as *Tuber indicum* as they are variations within a single species according to internal transcribed spacer and β-tubulin sequences[34]). Although they look alike,[35] *Tuber indicum* does not have the culinary value and taste as *Tuber melanosporum*[36] and is offered at low prices of about 60-200 €/kg.[37] Due to their morphological similarity, *Tuber indicum* and *Tuber melanosporum* are particularly difficult to distinguish and allow for especially high profit in the case of food fraud.[38] *Tuber indicum* has been observed as an invasive species that has now become native to Europe and North America.[31] Whether intentionally as an attempt to defraud or accidentally collected through lack of knowledge, mixing of truffle species during sale is observed.[24] Since clear identification of truffle fruiting bodies under the microscope is challenging and difficult and thus requires a lot of expertise and experience,[24] determining truffle species by PCR amplification or other genomics based methods[25,39-43] are possible, as well as proteomics approaches,[44-46] or with isotopolomics based methods.[47-50] But they all require a sophisticated laboratory environment and end up being time-consuming and expensive. Therefore, there is a requirement for further analytical methods to distinguish morphologically similar truffle species. Mannina *et al.*[51] have shown on *Tuber aestivum* that ^{1}H NMR of aqueous truffle extracts can be used to study their chemical composition (*Tuber aestivum*, named by Carlo Vittadini,[28,52] and *Tuber uncinatum* were summarized as *Tuber aestivum*, on the basis of molecular

biological findings that *Tuber aestivum* and *Tuber uncinatum* are the same species[53]). Although Creydt *et al.*[54] and Losso *et al.*[55] have recently shown that liquid chromatography electrospray ionization ion mobility quadrupole time of flight mass spectrometry (LC-ESI-IM-QTOF-MS) resp. direct analysis in real time mass spectrometry (DART-MS) and hydrophilic interaction liquid chromatography mass spectrometry (HILIC-ESI-MS) can make very good predictions in the classification of truffle species, it is reasonable to use NMR as an alternative analytical method, thanks to the particular advantages that this method brings.

In this study ^{1}H NMR spectroscopy of aqueous truffle samples and subsequent chemometrics were performed to show that the truffle species *Tuber aestivum*, *Tuber borchii*, *Tuber indicum*, *Tuber magnatum* and *Tuber melanosporum* can be distinguished with an overall accuracy of 99.5% from each other by means of NMR using a multivariate support vector machine (SVM) after 5-fold repeated nested cross validation. By selection and reduction of variables to only eight features with ranges of 0.05 ppm, a univariate method could be developed which, using this set of samples, can distinguish the species with an overall accuracy of 100%. In the direct comparison within the black truffles *Tuber melanosporum* and *Tuber indicum* betaine could be identified as a marker for *Tuber melanosporum*, which has been validated, building a two-class classification model. In addition, within the white truffles *Tuber magnatum* and *Tuber borchii*, ribonate could be identified as a marker exclusively found in *Tuber borchii*.

2. MATERIALS AND METHODS

2.1. Reagents and Materials.

Deuterated solvents chloroform-*d* (≥ 99.8%) and methanol-d_4 (≥ 99.8%) were purchased from Eurisotop (Saint-Aubin Cedex, France). Deuterium oxide (≥ 99.9%), sodium hydroxide-*d* (40% in D_2O), and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP-d_4) (≥ 99.0%) were purchased from Deutero (Kastellaun, Germany). Betaine (≥ 98.0%), potassium phosphate monobasic anhydrous (≥ 99.0%), potassium phosphate dibasic anhydrous (≥ 98.0%), sodium azide (≥ 99.5%), and

chlorosulfonic acid (≥ 99%), were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Potassium hydroxide pellets (≥ 85.0%) were purchased from Chemsolute (TH.Geyer, Renningen, Germany). D-(+)-ribono-1,4-lactone (≥ 97.0%) was purchased from Carbosynth Limited (Berkshire, United Kingdom). Ultrapure water was purified by a Sartorius arium pro apparatus (Sartopore 0.2 µm, UV). Choline chloride (≥ 99%), and N,N-dimethylformamide (≥ 99.8%) were purchased from fisher scientific (Schwerte, Germany).

2.2. Sample Acquisition.

In this study fruiting bodies from 80 truffle samples of five species which are used as foodstuff and are listed in the german "Leitsätze für Speisepilze und Speisepilzerzeugnisse",[56] originating from 11 countries were collected and analyzed between 2017 and 2020 shown in Tab. 1. The truffle samples were frozen and stored at −80 °C after arrival until further processing.

Table 1.: Truffle samples used in this work, 5 different species with different amounts from 11 countries.

Species (5)	Amount (80)	Origin (11 countries)
Tuber aestivum / *Tuber uncinatum*	28	Bulgaria 2, Italy 6, Iran 1, Moldova 1, Romania 12, Slovenia 1, Spain 1, unknown 4
Tuber albidum pico / *Tuber borchii*	7	Bulgaria 1, Italy 5, Spain 1,
Tuber indicum / *Tuber sinense*	12	China 12
Tuber magnatum	21	Bulgaria 2, Croatia 2, Italy 14, Romania 2, unknown 1
Tuber melanosporum	12	Australia 3, France 2, Italy 3, Spain 4

2.3. Sample Preparation.

Several fruiting bodies from one delivery of one region were combined to obtain at least 75 g of truffle sample. The fruiting bodies were cleaned with ultrapure water to remove remaining soil. Hereafter, the fruiting bodies were milled with the addition of dry ice using a knife mill (Grindomix GM 300, Retsch,

Haan, Germany) and freeze-dried for 48 h (Alpha 2-4 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), including a stirring step after 24 h. The lyophilized truffle samples were stored at −80 °C until further processing.

2.4. Polar Extraction for Classification.

Truffle samples were weighed in triplicate at 100 ± 0.9 mg. To the lyophilizate powder, 600 µL deuterated phosphate buffer (200 mM, 2 mM sodium azide, 0.5 mM TSP-d_4, pH = 7.0), 500 µL chloroform-*d*, and 400 µL methanol-d_4 were added on dry ice. After the addition of two metal beads (Ø = 2 mm), the samples were run for 3 minutes at 30 Hz in a Bead Ruptor 4 (Omni International, Kennesaw USA) and centrifuged for 10 minutes at 14,000 rpm and 4 °C (refrigerated centrifuge 5417 R Eppendorf, Wesseling-Berzdorf Germany). The supernatant was taken and 400 µL of methanol-d_4 was added to it to precipitate the remaining proteins from the extract. After mixing at 2,500 rpm for 5 seconds (Mixer Dig Vortex VWR, Radnor USA), the samples were centrifuged at 14,000 rpm and 4 °C for 2 minutes and 350 µL of the supernatant was transferred to an NMR tube (Ø = 5 mm) containing 350 µL of deuterated phosphate buffer. The sample was mixed with the buffer by inverting five times.

2.5. Polar Extraction for Metabolit Identification of *Tuber melanosporum*.

MeOH (60.0 mL) was added to 6.00 g of *Tuber melanosporum* lyophilisate and stirred for 60 min at room temperature. After the removal of the solvent under reduced pressure, MeOH (60.0 mL) was once again added to the truffle lyophilisate and stirred for 60 minutes at room temperature. The solvent was removed under reduced pressure. Subsequently, MeOH (40.0 mL), Millipore water (60.0 mL) and $CHCl_3$ (50.0 mL) were added to the reaction mixture and stirred for 20 hours at room temperature. The reaction mixture was centrifuged for 30 minutes at 8,000 rpm and 4.0 °C and the supernatant was separated. Furthermore the solvent was removed under reduced pressure and the polar extract was lyophilized at 1.00 mbar. The extract was dissolved in Millipore water (50.0 mL) and filtered through

3 kDa cut off spinfilter for 15 minutes at 14,000 rpm and 22 °C. To remove the present glycerin, the spinfilters were previously washed fifteen times with NaOH solution (100 mM) followed by one time with phosphate buffer (200 mM, 2 mM sodium azide, 0.5 mM TSP-d_4, pH = 7.0) for 15 minutes at 14,000 rpm and 22 °C.

2.6. Chromatography with C18.

For metabolite identification the polar extract of 6 g lyophilizate powder of *Tuber melanosporum* (see section 2.4.1) was purified with a VP 250/10 Nucleodur C18 Pyramid (Macherey-Nagel, Düren, Germany) with a Column Volume (CV) of 20 mL on a 1260 Infinity HPLC System (Agilent Technologies, Santa Clara, USA). The fraction size was 4 mL by a flow rate of 4 mL min^{-1}. The used gradient with the eluents B: acetonitrile and A: water, both solvents with the addition of 0.1% formic acid: 1.0 CV 0% B, 1.0 CV 0% - 10% B, 2.0 CV 10% - 25% B, 4.0 CV 25% - 75% B, 2.0 CV 75% - 95% B. Acetonitrile was removed from the fractions using rotary evaporator Laborota 4001 (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) before lyophilization. For mass spectrometry analysis, all fractions were separately dissolved in 1.00 mL Millipore water, homogenized and 40.0 µL were stored for further analysis. The residual solution of each fraction was lyophilized at 1.00 mbar and dissolved in 700 µL phosphate buffer (200 mM, 2 mM sodium azide, 0.5 mM TSP-d_4, pH = 7.0), resuspended, and 600 µL was used for ^{1}H NMR measurements.

2.7. Chromatography with HILIC.

For identification of highly polar metabolites the polar extract of 1 g (10 x 100 mg see section 2.4) lyophilizate powder of one *Tuber borchii*, one *Tuber magnatum*, and one *Tuber melanosporum* sample were fractionated with a POLYGOPREP 60-30 NH_2 silica gel Column (Macherey-Nagel, Düren, Germany) with a Column Volume (CV) of 71 mL on an Isolera One Purification Instrument (Biotage, Uppsala, Sweden). The fraction size was 20 mL by a flow rate of 20 mL min^{-1}. For *Tuber borchii* the following solvents and gradients were applied; eluents B: acetonitrile and A: water: 1.0 CV 90% B,

18.0 CV 90% - 50% B, 0.5 CV 50% - 0% B, 1.0 CV 0% B. For *Tuber magnatum*, and *Tuber melanosporum* eluents were B: acetonitrile and A: water applying the following gradients 2.0 CV 90% B, 13.0 CV 90% - 0% B, 1.0 CV 0% B. Acetonitrile was removed from the fractions using rotary evaporator Laborota 4001 (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) before lyophilization. For mass spectrometry analysis, all fractions were separately dissolved in 1.00 mL Millipore water, homogenized and 20.0 µL were stored for further analysis. The residual solution of each fraction was lyophilized at 1.00 mbar and dissolved in 700 µL phosphate buffer (200 mM, 2 mM sodium azide, 0.5 mM TSP-d_4, pH = 7.0), resuspended, and used for ^{1}H NMR measurements.

2.8. NMR Data Acquisition.

^{1}H NMR spectra for classification were acquired using a Bruker AVANCE III HD 400 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at 400.13 MHz. The pulse sequence noesygppr1d was used for acquisition of water-suppressed ^{1}H NMR spectra using the digitization mode baseopt. The spectra were recorded with a number of scans of 64 at 300 K. 65,536 complex data points were recorded in a spectral width of 8417.5 Hz. The transmitter frequency offset was set to 1930.6 Hz and the Receiver gain to 64.

^{1}H NMR spectra for metabolite identification after the chromatography of truffle extract and for spike in experiments were acquired using a Bruker AVANCE III HD 600 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at 600.13 MHz. The pulse sequence noesygppr1d and zgesgp were used for acquisition of water-suppressed ^{1}H NMR spectra using the digitization mode baseopt. The spectra were recorded with a number of scans of 64 or 128 at 298 K. 65,536 complex data points were recorded in a spectral width of 12295.08 Hz. The transmitter frequency offset was set to 2909.9 Hz and the Receiver gain to 1.

2.9. NMR Data Processing.

To obtain spectra, the free induction decays (FIDs) were Fourier-transformed with a multiplied e function and a line broadening factor of 0.3 Hz. An automatic zero order phase and baseline correction were performed with Topspin 4.0.8 (Bruker BioSpins, Rheinstetten, Germany). To minimize the data information for classification, the spectra were processed to a bucket table with the software Amix (Version 3.9.15, Bruker BioSpin, Rheinstetten, Germany). The 1D NMR simple rectangular buckets method with a size of 0.03 ppm was used from 0.6 to 9.0 ppm, with the positive intensity integration mode. The data was scaled to the reference region of TSP-d_4 from −0.05 to 0.05 ppm. The areas from methanol 3.30 to 3.33 ppm, and sides with no signals from 4.65 to 5.05 ppm and 6.20 to 6.48 ppm were excluded. A bucket table with 253 features for all 80 samples was obtained.

2.10. Principal Component Analysis

For the calculation of the Principal Component Analysis (PCA) with the software Amix (Version 3.9.15, Bruker BioSpin, Rheinstetten, Germany) the data from the bucket table were scaled to unit variance with a confidence level of 95% for each sample group. The minimum explained variance was set to 95%. The PCA score and loading plots were plotted using the software OriginPro 2021 (Version 9.8.0.200, OriginLab Corporation, Northampton, USA) with a confidence ellipse of 70%.

2.11. Multivariate Data Analysis.

The classification analysis and validation of the data were performed with the software Matlab R2020a (The MathWorks, Inc., Natick, USA) including the Apps Classification Learner and BoxPlotPro.[57] The data from the bucket table were classified in two two-class and one five-class model with a support vector machine (SVM) algorithm using a box constraint level of 1 and were validated with a repeated nested cross validation (RNCV).[58,59]

The following procedure was performed with the data set of the two black truffle species *Tuber melanosporum* against *Tuber indicum* as well as the white *Tuber magnatum* against *Tuber borchii*. Also,

the set of all five mentioned truffle species with the multiclass method *One-vs-All* were tested. Each data set was randomly separated into five equal parts whereby successive four of these parts were combined to a training set and the fifth used as a test set, to ensure that each part was the test set once. With the training sets, classification models were received by using the SVM algorithm, with a quadratic Kernel function and an internal five-fold cross validation. The obtained models were validated using the previously described left out test set. By repeating the whole process five times with again randomly remixed parts for each data set, a five-fold outer cross validation was carried out. The mean value of all 25 received test sets of one classification model was plotted graphically in a final confusion matrix to show the performance of the model.[58] To calculate the macro-F1 score the arithmetic mean of all F1 scores of the five classes is formed, which are formed from the harmonic mean of the class wise precision and sensitivity.

2.12. Analysis of Variance (ANOVA) with Bonferroni-Holm Correction.

To compare the variance of the group means in the buckets of all truffle species a one-way Anova was performed in R (R.oo Version v1.24.0), including the packages R.methodsS3 (Version v1.8.1), and R.utils (Version v2.11.0) using the aov() function. A Bonferroni-Holm correction were performed with the software Matlab R2020a (The MathWorks, Inc., Natick, USA) including the App Bonferroni-Holm Correction for Multiple Comparisons.[60,61]

2.13. ESI-MS Data Acquisition.

For mass analysis 20 µL of fractionated truffle extract were diluted 1:10 with 180 µL ultrapure H_2O and injected into an Agilent 6224 ESI-TOF mass spectrometer (Agilent Technologies Inc., Santa Clara, USA). Mass spectra were recorded in the positive and negative ion mode with in a mass range of *m/z* 110-3200. Data interpretation was performed using the Analysis software MestReNova v14.1.1-24571 (Mestrelab Research, S.L., Santiago de Compostela, Spain).

2.14. SCORE-metabolite-ID.

To find correlating molecular masses matching signals in ^{1}H NMR spectra during the identification of metabolites after chromatography, the SCORE-metabolite-ID (semi-automatic correlation analysis for reliable metabolite identification) app implemented in MATLAB was used.[62] Due to equal concentration change caused by chromatography, *m/z* values from MS and NMR signals at a specific chemical shift value, of the same molecule from several serial fractions were found with this app via semi automatically Pearson correlation.

2.15. Synthesis of ribonate.

The metabolite ribonate was synthesized by hydrolysis of 10 mg D-(+)-ribono-1,4-lactone in 1 mL deuterated phosphate buffer (200 mM, 2 mM sodium azide, 0.5 mM TSP-d_4, pH = 7.0) with 200 mM potassium hydroxide. The resulting ribonate was used for the spike in experiment without further purification.

2.16. Synthesis of choline-O-sulfate.

The metabolite choline-O-sulfate was synthesized according to Dupont *et al.*[63] A solution of 768 mg choline chloride (5.5 mmol, 1.1 eq.) in 10 mL dimethylformamide was cooled with ice to 0° C. Under stirring, 333 µL of chlorosulfonic acid (0.583 g, 5 mmol, 1 eq.) was added. The reaction mixture was stirred at 0 ° C for 2 hours. The formed HCl gas was neutralized over 1 M NaOH. The solvent was removed under reduced pressure. The resulting choline-O-sulfate was used for the spike in experiment without further purification.

3. RESULTS AND DISCUSSION

Extraction with water, methanol and chloroform is a suitable way to separate polar from non-polar constituents for NMR metabolomic studies.[64] Due to the fact that the signals of, for instance, amino acids and carbohydrates have a higher dispersion of NMR signals than fatty acids or lipids, using the polar fraction for data analysis is more promising.[65,66] In addition, methanol was used for protein precipitation to assure sample stability and that data analysis is not disturbed by a broad protein background. The stability of the sample was tested over a period of 1 month using a sample stored at room temperature and monitored daily by ^{1}H NMR.

Each sample was extracted threefold and the data presented are the average over all measurements. All analyses were additionally repeated using the first extract of each sample to validate that the mean values represent the actual biological variance and produced the same results. To minimize or exclude systematic errors within the truffle species, samples of different truffle species were randomly processed in an extraction run. Although no changes in signal intensities have been observed during room temperature storage, all ^{1}H NMR measurements were performed within the following day after extraction. Signal shifts attributed to pH-variations were not observed. Figure 1 shows a full ^{1}H NMR spectrum of the polar extract from *Tuber melanosporum* with annotations for peaks that were assigned after chromatographic separation, by means of 1D- and 2D NMR experiments, ESI-MS, using the SCORE-metabolite-ID app, and spike-in verification. 64 scans were recorded at 300 K using the 1D-NOESY pulse sequence for water suppression on a 400 MHz NMR spectrometer. After Data processing, a bucket table with 253 features of all 80 samples for classification was obtained.

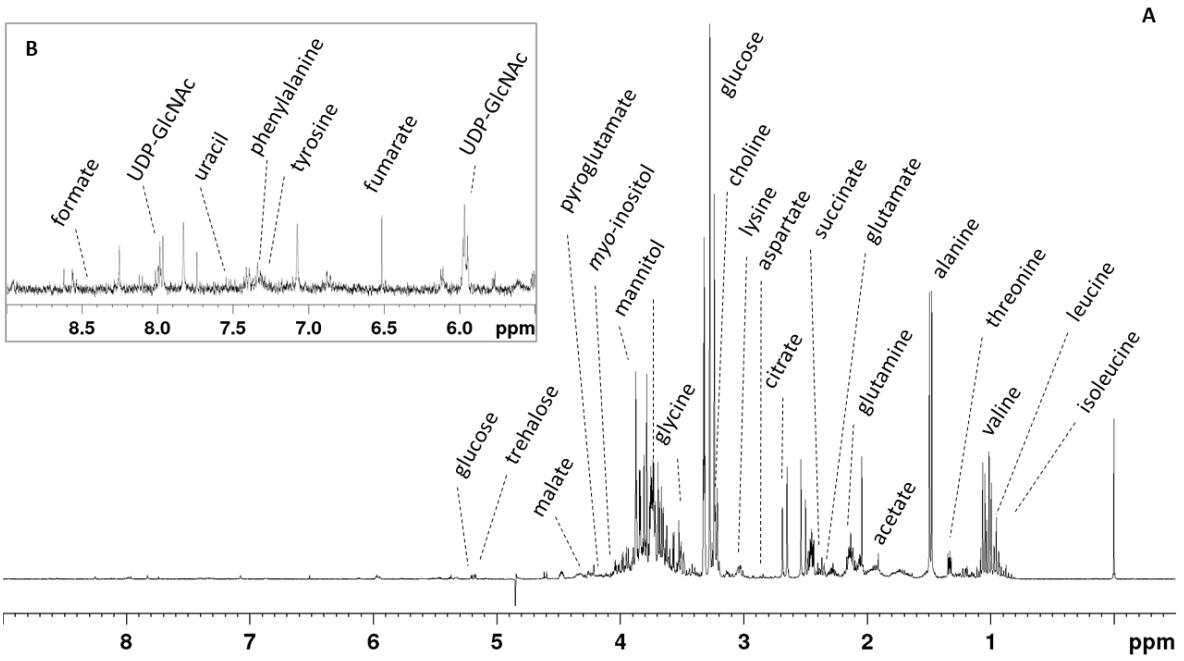


Figure 1.: (A) ^{1}H NMR spectrum of polar extract from *Tuber melanosporum* (sample 13, table S1) with annotated metabolites (table S2) (noesygppr1d, 400 MHz, 300 K, 64 scans, TSP-d_4 as internal standard). (B) Spread of the area between 5.5 and 9.0 ppm.

The most intense signals in the spectrum appear in the aliphatic region and belong to signals from amino acids such as alanine, leucine, isoleucine and valine. In addition, glucose and salts of organic acids such as citrate and succinate can also be observed. In the aromatic region, the signals showed a much lower intensity. A List of all identified metabolites is shown in the Supporting Information (table S2). The metabolites found in *Tuber melanosporum* are very similar to the metabolites described from *Tuber aestivum* by Mannina *et al.*[51]

3.1. Principal Component Analysis

Using the bucket table, a PCA was performed to visualize data variance. The first ten principal components represent 69% of the total variance, while 43 principal components account for 95% total variance. In PC1, there is a relatively large variance within the sample groups, which extends over the whole range of PC1. Whereas all five sample groups show a definite clustering in the following principal components. The two white truffle species separate across the second and third principal component, while the three black truffle species show variances on the axes of the second and fifth principal

component. Figure 2 shows the result of PCA as 2D plots in figure 2A (PC2 vs. PC3) and in figure 2B (PC2 vs. PC5). The PCA loadings plot of PC2 vs PC3, which is addressed in the discussion, is shown in the Supporting Information (figure S1). This result is promising for the setup of a classification model using a machine learning algorithm as it was subsequently applied.

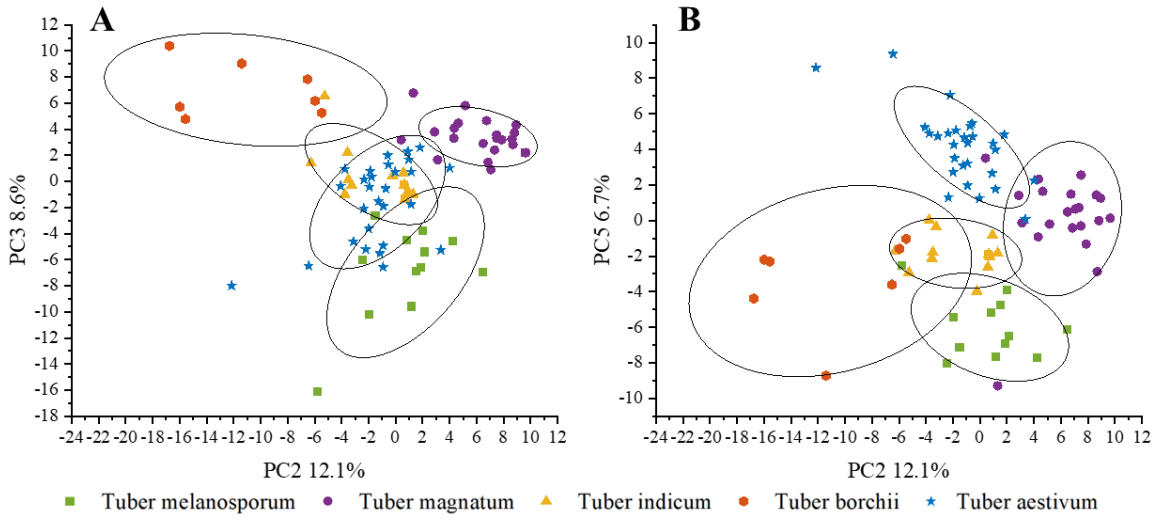
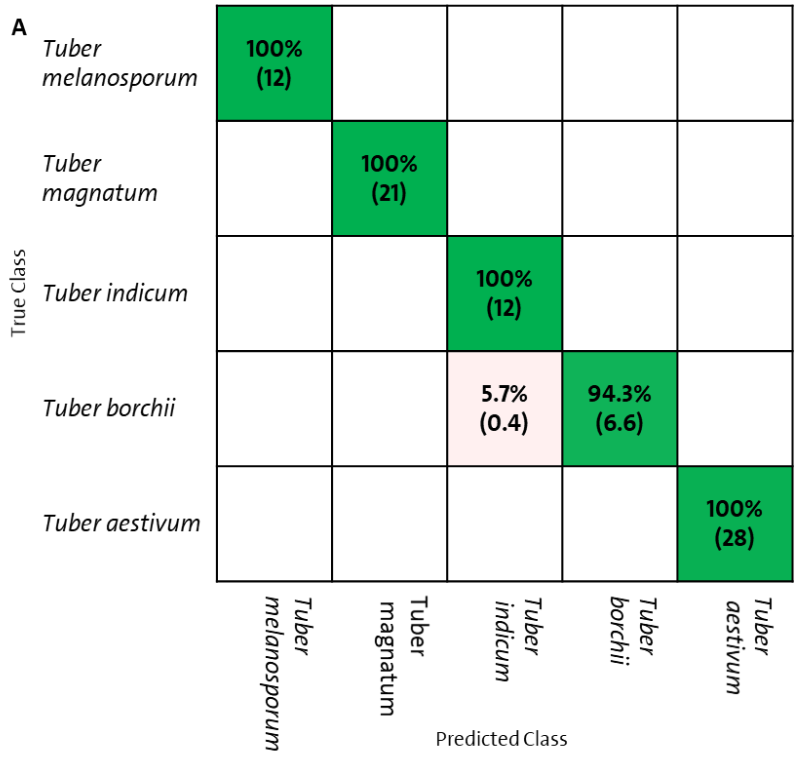


Figure 2: 2D PCA scores plot of (A) PC2 vs PC3 and (B) PC2 vs PC5 of the NMR analysis of polar extracts from 5 different truffle species. Explained variance PC2 = 12.1%; PC3 = 8.6%; PC5 = 6.7%. 3D confidence ellipsoid with a level of 70%.

3.2. Classification

In terms of simplified automated analysis, a five-class model was build testing several algorithms using the Classification Learner App in Matlab. Support Vector Machine (SVM) was finally chosen as one of the state-of-the-art classification methods giving the highest accuracies. The set of samples was randomly split into five parts, whereby successive four of these parts were combined to a training set and the fifth used as a test set for a five-fold cross validation. Validation of the data was further continued applying the repeated nested cross validation approach, repeating the whole process four times with randomly remixed splits for an outer five-fold cross validation. The final result is given by the mean over all 25 test sets. The two white truffle species *Tuber Borchii* and *Tuber magnatum* as well as the three black truffle species *Tuber aestivum*, *Tuber indicum* and *Tuber melanosporum* were compared. In the classification using all 253 features, an accuracy of 99.5% (± 0.69) was achieved, with a Macro-F1 score of 99.1%. Here, one *Tuber borchii* sample was miss-classified as *Tuber indicum* in two out of five

test sets. To reduce the amount of data and even enhance the classification accuracy, all areas without signal intensity were excluded. All remaining features were successively removed randomly in several runs and tested if the trained model exhibited an increased or decreased accuracy. During this procedure, the data was successfully reduced to eight features that allowed classification of the test set in a repeated nested cross validation with 100% accuracy. The corresponding confusion matrices before and after feature selection are shown in figure 3A and 3B, respectively. Box plots of the corresponding features for all five truffle species are shown in figure 4.



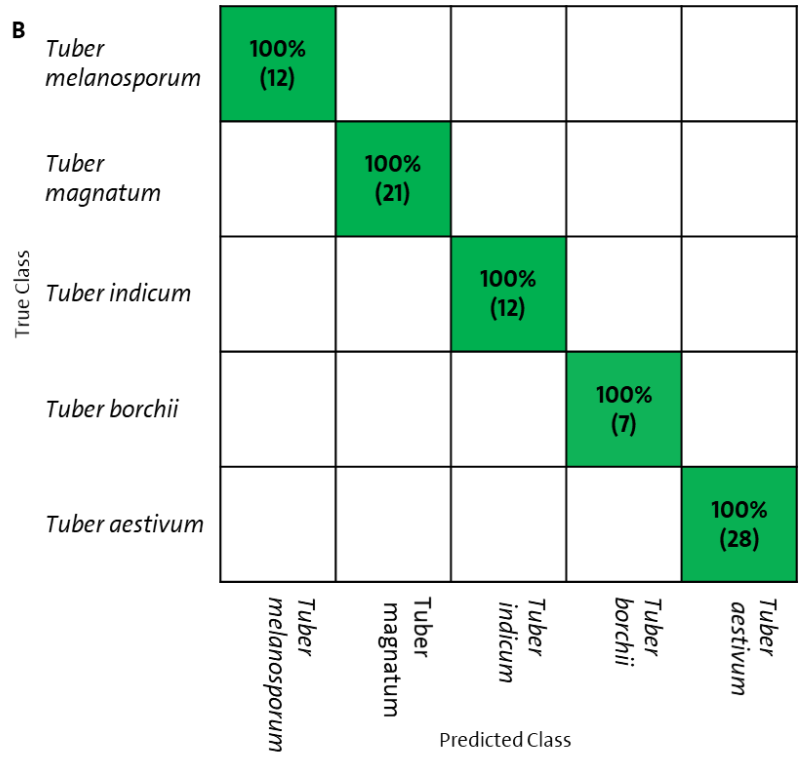


Figure 3: (A) Confusion matrix of the five-class model classification with all 253 features between *Tuber aestivum*, *Tuber borchii*, *Tuber indicum*, *Tuber magnatum* and *Tuber melanosporum* with confusion values shown in the accuracy in percentage and in count with an overall accuracy of 99.5% (± 0.69). (B) Confusion matrix of the five-class model classification with 8 features between *Tuber aestivum*, *Tuber borchii*, *Tuber indicum*, *Tuber magnatum* and *Tuber melanosporum* with confusion values shown in the accuracy in percentage and in count with an overall accuracy of 100%.

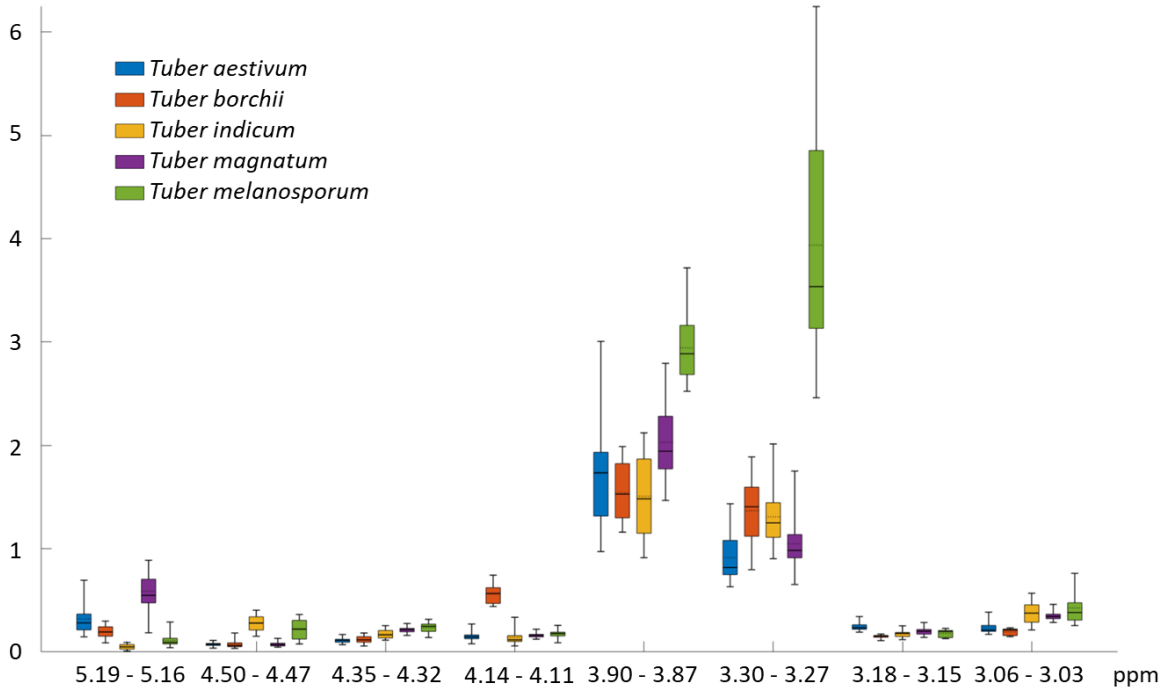


Figure 4: Box plots of the 8 selected features of the five-class model classification between *Tuber aestivum*, *Tuber borchii*, *Tuber indicum*, *Tuber magnatum* and *Tuber melanosporum*. The x axis shows the chemical shift of the feature, and on the y axis the distribution of the size of the integrals of each bucket over all samples is represented.

To confirm the relevance of the selected features independently, a one-way ANOVA was calculated using a Bonferroni-Holm correction for multiple comparisons. Five of the eight selected buckets are in the top ten of the smallest p-values and all eight are significant. A list with all p-values, p-values with Bonferroni-Holm correction and its ranking are shown in the Supporting Information (table S3). Furthermore, all 8 features have heavy loadings in the loading plot of PCA (PC2 vs PC3) and thus show impact on the clustering of the different truffle species (see figure S1 in the supporting information).

That the classification can be performed on all truffle species is a good result, but it is especially relevant to distinguish within the white or the black truffle species. Of particular importance is the differentiation of the morphologically similar *Tuber indicum* and *Tuber melanosporum*, which was achieved by support vector machine (SVM) with an accuracy of 100% when all 253 features were used. Again, the procedure of feature reduction has been applied. Two features were found in this procedure, both with significant differences in intensities in the boxplots. These features represent two singlets in the spectra of *Tuber*

melanosporum at 3.28 ppm and 3.87 ppm, each of which individually resulted in an unambiguously correct assignment, shown in a confusion matrix in figure 5A. Since in some spectra signals from other metabolites superimpose the singlet at 3.87 ppm, the singlet at 3.28 ppm should be used preferentially for distinction The corresponding box plot for the feature from 3.30 to 3.27 ppm is shown in figure 5B. A figure showing the distribution of intensities in the ^{1}H NMR spectra can be found in the Supporting Information in figures S2 and S3.

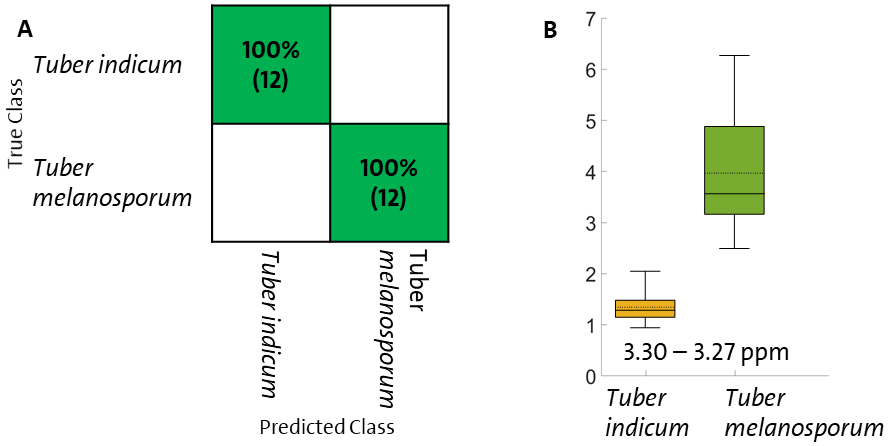


Figure 5.: (A) Confusion matrix of the two-class model classification between *Tuber indicum* and *Tuber melanosporum* with confusion values shown in the accuracy in percentage and in count. (B) Box Plot of the integration intensities of the feature from 3.30 to 3.27 ppm of *Tuber indicum* and *Tuber melanosporum*.

A similar result showed the classification model using all 253 features for differentiation of *Tuber borchii* and *Tuber magnatum* extracts. The SVM was able to assign both classes with 100% accuracy. The process of feature reduction was repeated in the same manner and revealed a doublet that was observed exclusively for *Tuber borchii* at 4.13 ppm. With only one out of 253 features again a 100% correct classification is possible, shown in confusion matrix in figure 6A. The box plots which represent the difference of the signal intensities of this feature from 4.14 to 4.11 ppm for all *Tuber borchii* and *Tuber magnatum* samples are shown in figure 6B. A figure showing the distribution of intensities in the ^{1}H NMR spectra can be found in the Supporting Information in figure S4 and S5.

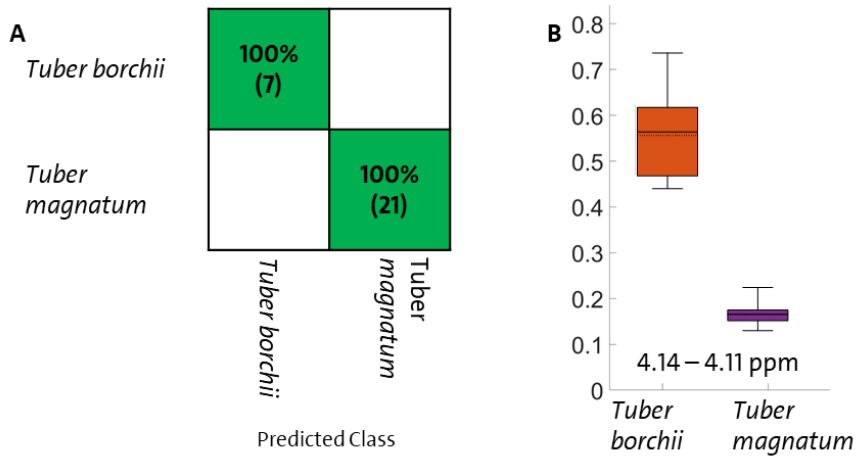


Figure 6.: (A) Confusion matrix of the two-class model classification between *Tuber borchii* and *Tuber magnatum* with confusion values shown in the accuracy in percentage and in counts. (B) Box Plots of the integration intensities of the feature from 4.14 to 4.11 ppm of *Tuber borchii* and *Tuber magnatum*.

In summary, a five-class model was successfully established for the distinction of all truffle species considered here. A high overall accuracy of 99.5% was already achieved when using all 253 features, with only 2 misclassifications of a single sample in two out of a total of 25 test sets. By feature selection down to eight features it was possible to eliminate the misclassifications. Furthermore, two two-class models could be trained, which only require a single feature for discrimination. Thus, the leap from a multivariate model to a univariate differentiation of the two white truffle species *Tuber borchii* and *Tuber magnatum* and the two black truffle species *Tuber indicum* and *Tuber melanosporum* is successful.

3.3. Metabolite Identification.

SVM combined with feature selection revealed single features by which both *Tuber indicum* and *Tuber melanosporum*, as well as *Tuber borchii* and *Tuber magnatum*, could be discriminated unambiguously. The next step was the identification of the corresponding biological markers. For the classification of the black truffles, *Tuber indicum* and *Tuber melanosporum*, two singlets were identified, with significantly higher intensities in all *Tuber melanosporum* extracts. In all spectra of the white truffle *Tuber borchii* a doublet was identified which is exclusive to this truffle species. It was not possible to

detect further signals assignable to the corresponding metabolites, neither by selective experiments, nor by 2D NMR experiments. Therefore, a chromatographic separation step was applied to reduce the complexity of the mixtures. Due to the high polarity of the metabolites in the aqueous extract, suitable retention and separation was achieved via aminopropyl-modified silica gel applying HILIC conditions. Through further NMR measurements, ESI-MS data, searching for correlations using the SCORE-metabolite-ID app, and subsequent spike-in experiments, the molecules could be identified.

In the case of the two singlets from *Tuber melanosporum*, results from the 2D NMR experiments HSQC and HMBC and a mass of 118.087 *m/z* obtained by ESI-MS in negative mode, gave reason for the assumption that betaine, could be the molecule of interest, which was confirmed by spike in experiments on the corresponding fraction. Figure S6 in the supporting information shows the increase in the expected signal after adding 100 µg betaine, confirming betaine as a concentration dependent biomarker for *Tuber melanosporum* compared to the other species analyzed in this work.

Classification and feature reduction revealed a doublet at 4.13 ppm unique to *Tuber borchii* extracts. To obtain further related signals of the molecule of interest, selective 1D TOCSY NMR was used. Additionally, H,H-COSY, HSQC and HMBC spectra were acquired and a mass of 165.040 *m/z* was obtained by ESI-MS in negative mode. The structure could be elucidated as ribonate. To confirm the proposal, commercially available D-(+)-ribono-1,4-lactone was saponified to ribonate using potassium hydroxide and used for spike-in experiments. Figure S7 in the supporting information shows the increase in the expected signal after adding 30 µg ribonate, confirming ribonate as an unique biomarker for *Tuber borchii* compared to all other species used in this work.

Three further features from the selected five-class model classification between *Tuber aestivum*, *Tuber borchii*, *Tuber indicum*, *Tuber magnatum* and *Tuber melanosporum* could be identified by analog approaches in the polar extract of *Tuber magnatum*. The feature at 5.18 ppm belongs to trehalose (spike in spectra are shown in figure S8 in the supporting information). The feature at 4.49 ppm is associated with choline-O-sulfate, which subsequently was synthesized according to Dupont *et al.*[63] Spike in

spectra are shown in figure S9 in the supporting information. The feature at 4.34 ppm is associated to glycerophosphorylcholine.

From these results, the question arises as to why ribonate is a unique metabolite only observed in the truffle species *Tuber borchii*. Ribonate is a building block in the pentose degrading pathway in some aerobic bacteria strains like *Pseudomonas*.[67] Due to the fact that bacteria associated with truffle fruiting bodies contribute to the truffle aroma and *Pseudomonas* was found in *Tuber borchii* one could assume that the presence of ribonate can be explained in this way.[68] The physiological function of betaine has been described both to protect cells from osmotic stress and as a catabolic source of methyl groups through transmethylation in biochemical pathways and can be found among nearly all living organisms.[69-74]

3.4. Conclusion.

We have shown that both, multivariate and univariate classification of different truffle species from polar truffle extracts works excellently, with 99.5% (±0.69) accuracy for multivariate and 100% accuracy for univariate classification. Furthermore, 6 of the 8 signals of the metabolites relevant for the univariate five class classification could be identified as trehalose, choline-O-sulfate, glycerophosphorylcholine, ribonate, and betaine. Among them are two molecules whose presence or high concentration can serve as direct markers for detection between the more expensive and the cheaper black or white truffles with 100% accuracy in classification. This is betaine as a marker for the expensive *Tuber melanosporum* at a higher concentration than in the less expensive *Tuber indicum*. And ribonate, which occurs only in the less expensive white truffle *Tuber borchii* and not in the most expensive *Tuber magnatum*. With these NMR results, a new analytical tool is available, which is faster and cheaper than genetic analysis and more reliable than microscopy to distinguish the truffle species in question 100% correctly. In distinguishing the two white truffle species, the use of statistical software can be completely omitted and a simple yes/no query for the presence of the doublet of ribonate is sufficient for the analysis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

List of truffle samples; list of identified metabolites; 2D PCA loading plot; Figures of stacked ^{1}H NMR spectra showing the relevant peaks; Figures of stacked ^{1}H NMR spectra showing the spike-in experiments; list of ANOVA p-value ranking with Bonferroni-Holm correction

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

COSY, correlation spectroscopy; ESI-MS, electrospray ionization-mass spectrometry; ESI-TOF, electrospray ionization-time of flight; FID, free induction decay; HMBC, heteronuclear multiple-bond correlation spectroscopy; HSQC, heteronuclear single-quantum coherence; NMR, nuclear magnetic resonance; PC, principal component; PCA, principal component analysis; SVM, support vector machine; TSP-d_4, 3-(trimethylsilyl)propionic--,2,3,3-d_4; TOCSY, total correlation spectroscopy

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