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# No more nutmegging with nutmeg: Analytical fingerprints for distinction of quality from low-grade nutmeg products

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## ABSTRACT

Quality nutmeg is characterized by a delicate aroma. Next to quality nutmeg, other – lower - grades exist on the market, such as extracted material (spent) or ground shell or dried fruit pulp. Strong fluctuations in the price of nutmeg lead to rapid changes in market dynamics and marketing opportunities, and unfortunately results in illegal commingle of ground quality nutmeg with low-grade material. In this study, we examined fingerprints of volatile and non-volatile compounds of high quality and low-grade nutmeg material by Proton Transfer Reaction Mass Spectrometry and Flow Infusion ElectroSpray Ionization Mass Spectrometry, respectively. They were compared with data from classical measurements such as total ash, acid insoluble ash, moisture and volatile oil contents. Differences in composition were examined by univariate and multivariate statistical methods. Furthermore, one-class classification models for quality nutmeg were estimated using different algorithms and their performances were examined with quality nutmeg and low-grade material, as well as mixtures thereof. Distinct differences between quality nutmeg and low-grade nutmeg samples were observed for both their volatile and non-volatile fingerprints. Intensities of volatiles and non-volatiles are highly correlated, but this phenomenon diminishes gradually and even reverses with rising molecular mass of the non-volatiles. Results showed that both techniques allowed a nearly 100% correct prediction of quality nutmeg and low-grade nutmeg samples. Therefore, both approaches are promising and with further database extension, they may become a valuable addition to the analytical authentication toolbox in addition to the classical methods and help to detect future 'nutmeggers'.

## 1. Introduction

Since ancient times, people used spices to create a more desirable taste of their foods. Specific types of spices were used in different cuisines around the world to create characteristic flavours. The flavour of foods was enhanced but spices were also applied to preserve foods. In some cases, spices were even used to mask spoilage or off-flavours in products. This use continued for centuries without any real understanding of the processes behind it. Because of their importance for tasty foods, spices have become valuable plant products (Ockerman & Basu, 2014). From early history, entire economies of regions were based on the trade of spices and the region that could control spice trade dominated as a world power. Although this has changed, and spices account now for less than 0.1% of world trade, also in current times for

some economies spices are fairly important, e.g. cloves for Tanzania, vanilla for Madagascar, and nutmeg and mace for Grenada (Risch, 1997).

Spices and herbs come from a number of different parts of plants. This include the aril (mace), bark (e.g. cinnamon), berries (e.g. peppercorn), bulbs (e.g. garlic), flower (saffron), leaves (e.g. bay leaves), roots (e.g. ginger) and seeds (e.g. nutmeg) of a plant (Embuscado, 2018). Spices are commonly grown in tropical regions of the world. This can present challenges to companies that are trying to find a consistent source of spices both from a quality and supply perspective. With the growth in the use of spices, there has been continuous research into the active components of spices, both for its flavour (e.g. Silvis, Luning, Klose, Jansen, & van Ruth, 2019) and other bio-active compounds (e.g. Tajkarimi, Ibrhaim, & Cliver, 2010), but also attention was

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paid to the microbiological and chemical hazards (e.g. Banach, Stratakou, van der Fels-Klerx, den Besten, & Zwietering, 2016).

For the vast majority of applications, the spice is processed before it is used in a product. Generally processing usually involves some means of particle size reduction. The process of grinding breaks down some of the cell structure to make the volatile oils in the plant more readily released when used than if the spices were left in the intact form. In addition to grinding or comminution of the plant part, a means of bacterial reduction is also often needed. Different methods employed to reduce the bacterial load on spices include steam treatment, irradiation and ethylene oxide (Embuscado, 2018; Risch, 1997).

Nutmeg (*Myristica fragrans*) is a well known spice from the nutmeg tree. The nutmeg is the kernel inside the fruit pit, and mace is the bright red or purple lacy covering on the pit (the aril). Nutmeg and mace are dried and sold whole or ground, both are strongly aromatic, and have a long history (Periasamy, Karim, Gibrelibanos, Gebremedhin, & Gilani, 2016).

The nutmeg tree is indigenous to the Moluccas in Indonesia – the Banda islands – and many battles were fought because of the nutmeg. Nutmeg has been important for the Dutch society for centuries. In the early days, Europeans believed that nutmeg had the power to prevent and cure particular diseases. As a result, the spice was worth more than its weight in gold. Nutmeg was traded over land by Arabs, but their monopoly was broken by the Europeans. After wars with the Portuguese and the English, the Dutch materialised a nutmeg monopoly with the Treaty of Breda in 1667 when the Banda islands came under their control. At that moment the 10 Banda Island were home to all the world's nutmeg trees. The Dutch monopolised the global nutmeg market from the 17th until the 19th century selling nutmeg in Europe at 300 times the production cost. It was only then when the British managed to obtain seedlings and figured out how to cultivate nutmeg in Malaysia and India (Neatorama, 2018; ThoughtCo., 2018; Van Gils & Cox, 1994).

For the historical trade, nutmeg has been a common culinary spice in the Netherlands for centuries. It is used to flavour various cooked vegetables, such as asparagus cauliflower, Brussels sprouts, French beans, and spinach. It is also used in mashed potatoes and sauces (e.g. bechamel sauce). Moreover, it is applied in sweet bakery products, for instance in the traditional December pastries. Because of the strong flavour, small quantities are sufficient (Lute, 2017). In other Western countries, nutmeg is mostly reserved for sweet dishes, such as cookies, pies, puddings, spices cakes, and stewed fruits. On the contrary, in the Asian cuisines, nutmeg is a traditional ingredient of a variety of savoury dishes, such as curries (Cook's info, 2018).

However, nutmeg has a dark side as well, especially when it comes to its health effects. Although a low dose of nutmeg does not present psychological or neurological effects, excessive use may cause hallucinations. The first case of nutmeg intoxication concerned a pregnant English woman, who ingested 10–12 nutmeg nuts (Cushny, 1908). The effects are probably based on the conversion of myristicin into 3-methoxy-4,5-methylenedioxyamphetamine. Unduly use of the active compound myristicin can be dangerous, an intake of 5 g or more by an adult results in distinct clinical symptoms, such as stomach pains, vomiting, dizziness, agitation and change of consciousness. Myristicin is generally known in literature as a strong hallucinogen (Kelly, Gavin, Clarke, Lane, & Larkin, 2003; Petrykiv, de Jonge, Michielsen, & Arts, 2016).

A well producing nutmeg tree may give on average an annual production of 14–22 kg green nutmegs, which corresponds to 7–11 kg of shelled, dry nutmegs. The proportion of dried shelled nutmeg to dried mace is approximately 20: 3. During drying nutmeg loses about 25% of its weight. Yields vary greatly between trees, and between plantations or field locations (Marcelle, 1995). The global production of nutmeg and mace is estimated at 10,000–12,000 and 1500 to 2000 tons, respectively. Indonesia and Grenada dominate production and exports of both products with a world market share of 75% and 20%,

**Table 1**  
Examples of reports on adulteration of nutmeg and mace.

Ingredient	Type of adulteration	Reference
Nutmeg	Coffee husks	2004 <a href="#">ASTA (2011)</a>
Nutmeg mace	Bombay mace	2016 <a href="#">Food fraud risk information (2016)</a>
Mace ( <i>Myristica fragrans</i> )	Mace ( <i>Myristica malabarica</i> )	2016 <a href="#">USP database (2018)</a>

respectively. The key import markets are the EU, the USA, Japan and India. The Netherlands is a major re-exporter in the EU.

Whole nutmegs can be grouped in three quality categories: sound nutmegs, which are mainly used for grinding, substandard nutmegs, which are used for grinding and extraction, and poor quality nutmegs, which are used for extraction only (FAO, 1994). High-quality nutmeg should be oil-rich, whole, unbroken and without stalks, and must not contain any woody, brittle nutmeg. It should be reddish-brown to brown in colour and the head should be light brown and closed. Nutmeg, whether whole or crushed/ground, is often intentionally adulterated, for example with other members of the genus *Myristica fragrans* or by inferior parts of nutmeg (shell, fruit pulp) or extracted product (spent) (Table 1). The latter only in its ground form, obviously. The spice industry in general is highly vulnerable to fraud compared to other chains (van Ruth, Luning, Silvis, Yang, & Huisman, 2018). Unintentional contamination, due for example to fertiliser spill over or insects, may also be encountered. It is also at risk of contamination with mycotoxins (aflatoxin, ochratoxin and others) (ESA, 2015). Therefore, product quality is a key issue for buyers of nutmeg in Europe and so is adulteration. Some examples of adulteration are listed in Table 1. In fairness, no one likes being nutmegged. This interesting term has its roots in a sharp practice used in nutmeg exports between America and England where wooden replica nutmegs were mixed into the sacks of real nutmeg being shipped to England (Lohman, 2017). Those would not have been difficult to detect, but admixtures of good quality nutmeg and lower grade, extracted material are. DNA-based methods, how useful for other nutmeg authentication applications (Tallei & Kolondam, 2015), will not help to distinct grades and although there are many methods for spices described, there are very few aiming at authentication of nutmeg (Galvin-King, Haughey, & Elliott, 2018; Reinholds, Bartkevics, Silvis, van Ruth, & Esslinger, 2015).

In this study, we characterise quality nutmeg and low-grade nutmeg material by their broad composition, i.e. their volatile and non-volatile fingerprints. The fingerprints are measured by Proton Transfer Reaction Mass Spectrometry (PTR-MS) and Flow Infusion ElectroSpray Ionization Mass Spectrometry (FI-ESI-MS). The sets are evaluated by univariate and multivariate statistical methods, and the two sets of fingerprints are correlated. Sets are also correlated with classical analyses such as total ash, acid insoluble ash, moisture, and volatile oil content data. Furthermore, classification models for quality nutmeg - allowing for verification of the identity of nutmeg - are estimated and their performances examined with quality and low-grade material, as well as mixtures thereof.

## 2. Materials and methods

### 2.1. Sample material

Fifty ground quality nutmeg samples from Indonesia from the 2009–2014 harvests were provided by a European company that processed the nutmegs itself. Furthermore, seven samples of low-grade material were provided by two European spice companies, they comprised spent samples of various origins. For the characterization, the quality nutmeg samples and low-grade samples compositions were compared. For the development of classification models, five pooled samples were randomly prepared from 10 quality nutmeg samples each.

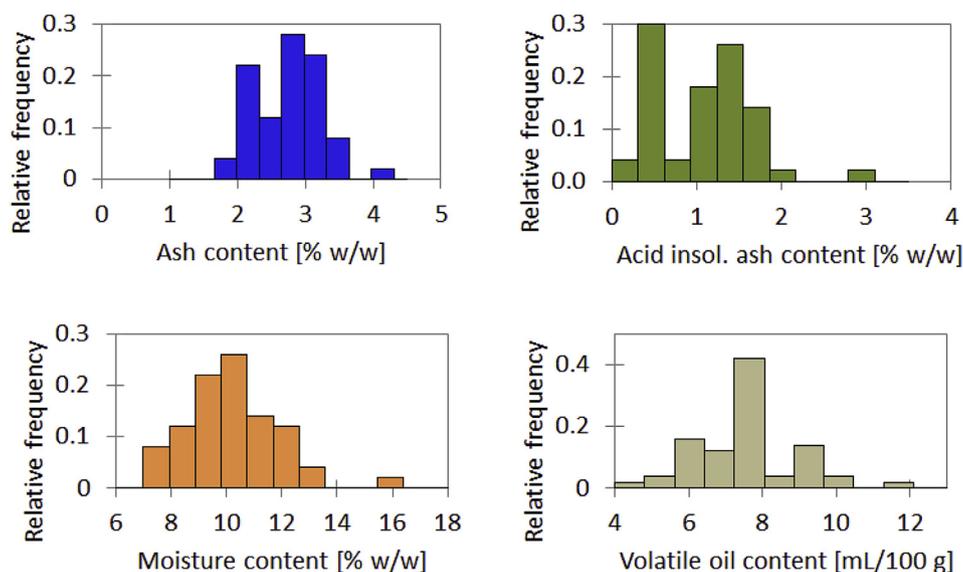


Fig. 1. Frequency distributions of total ash, acid insoluble ash, moisture, and volatile oil contents of quality nutmeg samples.

The five pooled samples were subsequently mixed 1:1 with each low-grade sample, resulting in 35 additional samples. The 50 quality samples were assigned to the baseline group, which in turn was used to develop the model. The pooled samples and the low-grade samples, as well as the mixed samples were used as challenge group, i.e. to challenge the classification models.

## 2.2. Analysis of the gross composition of authentic nutmeg

The quality nutmeg samples were analysed for their gross composition according to ISO standard 6577:2002 (2002), which comprised measurements of total ash content according to ISO standard 928:1997 (1997), acid-insoluble ash content according to ISO standard 930:1997 (1997), moisture content according to ISO standard 939:1980 (1980) and volatile oil content according to ISO standard 6571:1984 (1984).

## 2.3. Fingerprints of volatile compounds: PTR-MS

### 2.3.1. Sample preparation

The nutmeg samples were collected in powdered form (industrially ground) and did not require further grinding steps. The sample material (40 mg) was transferred into a 250 mL flask (Schott Duran bottles, Germany), which was capped and equilibrated in a water bath at 25 °C for 30 min. Preliminary experiments showed that 30 min were sufficient for equilibration (Silvis et al., 2019).

### 2.3.2. PTR-MS analysis

The volatile fingerprint of nutmeg samples were measured in triplicate using a high sensitivity PTR-MS instrument (Ionicon Analytik, Innsbruck, Austria). The order of sample and triplicate measurements were randomised to account for possible memory affects. All measurements were carried out under drift tube conditions of 120–140 Td (Td = Townsend; 1 Td = 10–17 V cm<sup>2</sup> mol<sup>-1</sup>) over a mass range of  $m/z = 26$  to  $m/z = 160$  and a dwell time of 0.2 s/mass, giving a cycle time of 32 s. The flasks with the samples were connected to the PTR-MS inlet flow that was heated to 60 °C via Teflon (0.25 mm) tubing and headspace air was sampled at a flow rate of 50 mL/min. Masses were analysed in a quadrupole mass spectrometer and detected as ion counts per second (cps) by a secondary electron multiplier. Sample measurements were performed in 5 cycles resulting in an analysis time of 3.0 min. Furthermore, background air scans of five cycles were conducted from an empty bottle before each sample measurement. Preliminary analyses had shown that no peaks were observed for nutmeg beyond the  $m/z$

26–160 mass range.

### 2.3.3. PTR-MS data treatment

The headspace concentrations of the compounds during the cycles #2, #3 and #4 were calculated as described by Hansel et al. (1995). Subsequently, background and transmission corrections were applied. Then, the three averaged mass spectra of the three replicates of each sample were averaged to obtain a mean mass spectrum per sample. In this manner, a data matrix comprising mean mass spectra for all samples was compiled.  $m/z$  21 (H<sub>3</sub>O<sup>+</sup>), 32 (O<sub>2</sub><sup>+</sup>) and  $m/z$  37 (water, oxygen and water cluster ion, respectively), that are associated with the PTR-MS ion source, were removed from the data set.

## 2.4. Fingerprints of non-volatile compounds: FI-ESI-MS

### 2.4.1. Sample preparation

Two-hundred mg of sample was extracted in 5 mL of 100% ethanol (Emsure, Darmstadt, Germany). Samples were agitated head-over-head at 20 °C for 1 h and centrifuged at 3000 rpm for 5 min at 4 °C. The clear layers were collected and kept at 4 °C for approximately 24 h. Subsequently the clear layers were collected and diluted 100-fold in 100% ethanol (Emsure, Darmstadt, Germany). The resulting solution was filtered using a Single StEP Filter Vial (Thomson Instrument Company, Oceanside, CA, USA) equipped with a 0.45 μm filter base (GE Healthcare, UK).

### 2.4.2. FI-ESI-MS analysis

For FI-ESI-MS, an Orbitrap mass spectrometer was used. This technology drives high-resolution, accurate mass data. A volume of 1 μL of samples was injected and transferred to the mass spectrometer using a mixture of methanol/water/formic acid (90:10:0.1) at a flow of 200 μL/min. A single stage Orbitrap Mass Spectrometer (Thermo Scientific, Bremen, Germany) was coupled to an Accela LC system (Thermo Scientific) and the injection part of the LC system auto sampler was connected directly to the HESI-II electrospray source of the mass spectrometer using ~75 cm PEEK tubing with an internal diameter of 0.13 mm. The HESI-II electrospray source was used in positive ionization mode with the following parameters: Spray voltage 3.5 kV; sheath gas 15 arbitrary units (au); auxiliary Gas 6 au; heater temperature 375 °C; capillary temperature 300 °C. The MS was used at a resolving power of 105, resulting in a scan rate of 1 Hz. Mass spectra in the range of  $m/z$  75–750 were recorded. To minimize sample carry-over, a blank solvent was injected after every sample. Each sample was injected

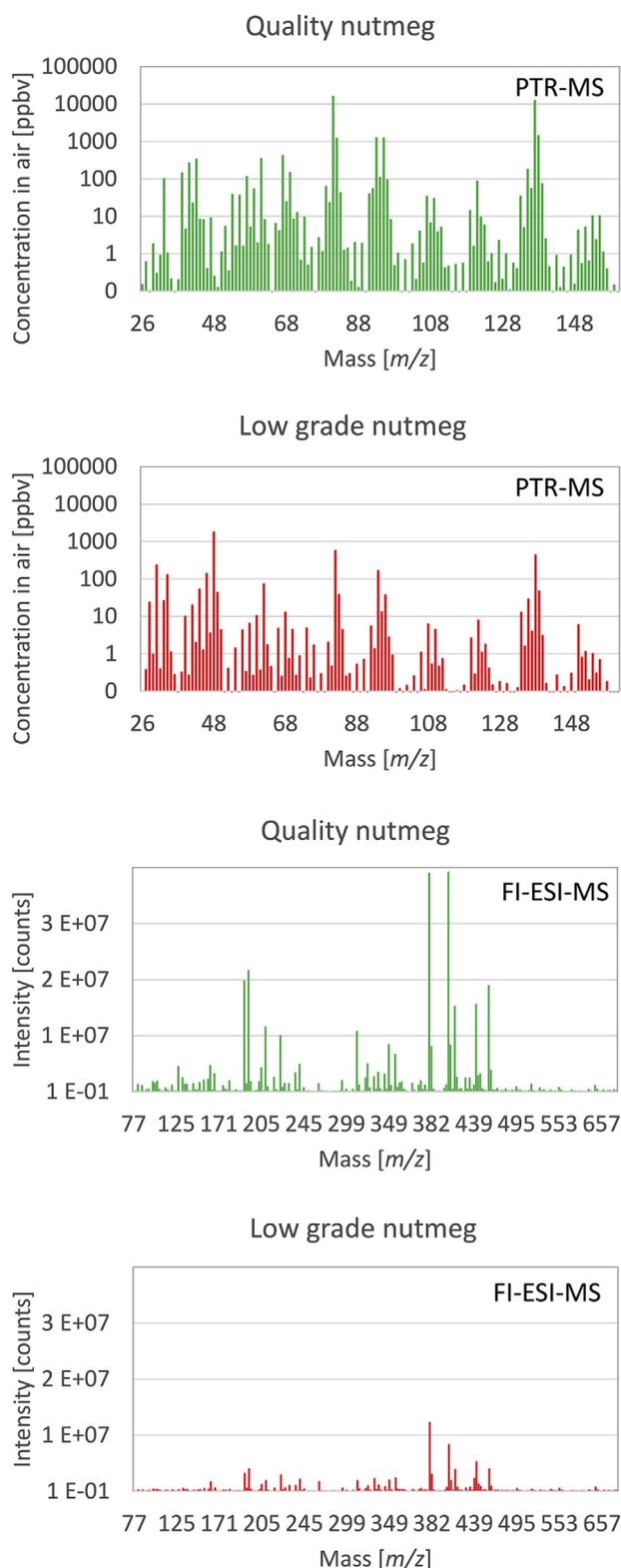


Fig. 2. Mean PTR-MS (log scale) and FI-ESI-MS fingerprints of quality nutmeg ( $n = 50$ ) and low-grade ( $n = 7$ ) material.

twice.

#### 2.4.3. FI-ESI-MS data treatment

The raw data produced by the mass spectrometer were converted to the NetCDF format using the file converter build in the Xcalibur software (version 3.0, Thermo Scientific). Xcalibur was also used to control

the mass spectrometer. The NetCDF-files were then converted by in-house adaptation of the XCMS package using R 3.2.2 software (the R Foundation for Statistical Computing, Vienna, Austria). Peaks were found within each sample and the files pre-sorted into different classes suitable for grouping; the peak groups were generated taking into account an error of 5 ppm for the full mass range. In this phase, the signal-to-noise cut off was defined. Once the groups were created, peaks were aligned across all samples using overlapping  $m/z$  bins and calculating the smoothed peak distribution. After peak grouping, intensity values were produced in the peak groups that did not include peaks from every sample. For those missing samples, raw data were integrated in the peak group region.

## 2.5. Statistical analyses

### 2.5.1. Characterization

**2.5.1.1. Univariate comparisons.** Means and standard deviations were calculated across each sample group, coefficients of variation were subsequently calculated by dividing the standard deviation of each mass by the mean of each mass (separately for the quality and low-grade nutmeg groups and techniques) and multiplication by 100%. Overall coefficients of variance (CV%) were determined by averaging the coefficients of variation across all masses for each samples group and technique. In order to investigate the significance of differences between the quality nutmeg and the low-grade group, the mass peak intensities from the PTR-MS and the FI-ESI-MS analyses were subjected to Mann-Whitney tests (XLstat 2017; Addinsoft, New York, USA) because of their non-normality nature. A significance level of  $p = 0.01$  was used throughout the study.

**2.5.1.2. Multivariate comparisons.** Multivariate analysis was conducted using Pirouette 4.0 Software (Infometrix, Seattle, WA, USA) and R 3.2.2 (the R Foundation for Statistical Computing). Principal Component Analysis (PCA) was used to visualize any natural clustering of the samples. Various data pre-treatments were considered, such as auto-scaling, mean centering, log10 transformation, and normalisation to the total mass peak intensities.

**2.5.1.3. Correlation analysis.** Correlation between the total mass intensity of volatile and non-volatile compounds of the nutmeg samples on the one hand, and volatile oil, moisture and (acid insoluble) ash contents of the samples on the other hand were assessed by Pearson correlation tests resulting in Pearson correlation coefficients ( $r$ ).

Furthermore, the relationship between fingerprints of volatile compounds and non-volatile compounds was determined by computing the Pearson correlation coefficients of PTR-MS concentrations versus FI-ESI-MS mass intensities.

### 2.5.2. Classification

In this study, we aim to detect different kinds of lower grade material in a single step. This approach is called one-class classification (OCC; Khan & Madden, 2014) and is related to (multivariate) classification in the sense that it can be used to objectively classify samples into “normal” and “deviating” samples. “Normal” samples (quality nutmeg) - covering the natural variation - serve as the basis for the determination of critical limits for classification, where any sample not fitting the multivariate natural variation of the quality nutmeg is considered as deviation (low-grade nutmeg).

The data set was screened for consistency using PCA and a few outliers were identified. Where the time between analyses for the authentic baseline samples and the challenge series was larger than three months, some consistent differences between pools and their parent baseline samples were observed, probably because of the combination of sample ageing and some analytical systemic error. These were corrected for using the medians of pools and baseline samples as series

**Table 2**

Comparison mass-to-charge ratio intensities of quality nutmeg and low-grade nutmeg measured by PTR-MS (mean of all samples per group  $\pm$  standard deviation). P-values indicate significance of differences (Mann-Whitney test).

Mass [m/z]	Quality nutmeg n = 50 [ppbv]	Low-grade nutmeg n = 7 [ppbv]	p-value	Mass [m/z]	Quality nutmeg n = 50 [ppbv]	Low-grade nutmeg n = 7 [ppbv]	p-value	Mass [m/z]	Quality nutmeg n = 50 [ppbv]	Low-grade nutmeg n = 7 [ppbv]	p-value
26	0 $\pm$ 0 <sup>a</sup>	0 $\pm$ 0	0.512	72	1 $\pm$ 0	0 $\pm$ 0	< 0.0001	116	0 $\pm$ 0	0 $\pm$ 0	< 0.0001
27	1 $\pm$ 0	24 $\pm$ 26	0.543	73	9 $\pm$ 6	5 $\pm$ 2	0.007	117	1 $\pm$ 0	0 $\pm$ 0	< 0.0001
28	0 $\pm$ 0	1 $\pm$ 1	0.024	74	1 $\pm$ 0	0 $\pm$ 0	0.002	118	0 $\pm$ 0	0 $\pm$ 0	< 0.0001
29	2 $\pm$ 1	237 $\pm$ 259	0.551	75	1 $\pm$ 1	2 $\pm$ 1	0.840	119	14 $\pm$ 5	3 $\pm$ 3	< 0.0001
30	0 $\pm$ 1	0 $\pm$ 1	0.408	76	0 $\pm$ 0	0 $\pm$ 0	0.332	120	2 $\pm$ 1	0 $\pm$ 0	< 0.0001
31	1 $\pm$ 0	26 $\pm$ 24	0.002	77	3 $\pm$ 1	0 $\pm$ 0	< 0.0001	121	86 $\pm$ 32	8 $\pm$ 8	< 0.0001
33	101 $\pm$ 40	129 $\pm$ 115	0.877	78	1 $\pm$ 0	0 $\pm$ 0	< 0.0001	122	10 $\pm$ 3	1 $\pm$ 1	< 0.0001
34	1 $\pm$ 1	1 $\pm$ 2	0.123	79	63 $\pm$ 26	2 $\pm$ 3	< 0.0001	123	6 $\pm$ 2	2 $\pm$ 2	< 0.0001
35	0 $\pm$ 0	0 $\pm$ 0	0.934	80	23 $\pm$ 10	0 $\pm$ 1	< 0.0001	124	1 $\pm$ 0	0 $\pm$ 1	0.038
36	0 $\pm$ 0	0 $\pm$ 0	0.025	81	15827 $\pm$ 5515	569 $\pm$ 961	< 0.0001	125	1 $\pm$ 0	0 $\pm$ 0	< 0.0001
38	0 $\pm$ 0	0 $\pm$ 0	0.177	82	1215 $\pm$ 457	38 $\pm$ 65	< 0.0001	126	0 $\pm$ 0	0 $\pm$ 0	< 0.0001
39	144 $\pm$ 51	10 $\pm$ 11	< 0.0001	83	43 $\pm$ 16	4 $\pm$ 7	< 0.0001	127	2 $\pm$ 1	0 $\pm$ 0	< 0.0001
40	5 $\pm$ 2	0 $\pm$ 0	< 0.0001	84	1 $\pm$ 0	0 $\pm$ 0	< 0.0001	128	0 $\pm$ 0	0 $\pm$ 0	< 0.0001
41	266 $\pm$ 83	20 $\pm$ 21	< 0.0001	85	1 $\pm$ 0	0 $\pm$ 0	< 0.0001	129	1 $\pm$ 0	0 $\pm$ 0	< 0.0001
42	23 $\pm$ 25	2 $\pm$ 1	< 0.0001	86	0 $\pm$ 0	0 $\pm$ 0	< 0.0001	130	0 $\pm$ 0	0 $\pm$ 0	< 0.0001
43	337 $\pm$ 174	53 $\pm$ 30	< 0.0001	87	2 $\pm$ 1	1 $\pm$ 1	< 0.0001	131	1 $\pm$ 0	0 $\pm$ 0	< 0.0001
44	8 $\pm$ 4	1 $\pm$ 1	< 0.0001	88	0 $\pm$ 0	0 $\pm$ 0	< 0.0001	132	0 $\pm$ 0	0 $\pm$ 0	0.000
45	8 $\pm$ 2	139 $\pm$ 132	0.410	89	2 $\pm$ 1	1 $\pm$ 0	0.002	133	35 $\pm$ 10	13 $\pm$ 19	0.001
46	0 $\pm$ 0	4 $\pm$ 3	0.332	90	0 $\pm$ 0	0 $\pm$ 0	0.003	134	5 $\pm$ 2	2 $\pm$ 2	< 0.0001
47	9 $\pm$ 5	1802 $\pm$ 1964	0.424	91	40 $\pm$ 12	5 $\pm$ 7	< 0.0001	135	178 $\pm$ 44	29 $\pm$ 37	< 0.0001
48	0 $\pm$ 0	44 $\pm$ 48	0.512	92	54 $\pm$ 19	1 $\pm$ 2	< 0.0001	136	55 $\pm$ 18	4 $\pm$ 6	< 0.0001
49	0 $\pm$ 0	4 $\pm$ 5	0.481	93	1262 $\pm$ 382	167 $\pm$ 232	< 0.0001	137	12373 $\pm$ 4555	434 $\pm$ 750	< 0.0001
50	1 $\pm$ 0	0 $\pm$ 0	< 0.0001	94	108 $\pm$ 32	13 $\pm$ 18	< 0.0001	138	1432 $\pm$ 548	48 $\pm$ 82	< 0.0001
51	5 $\pm$ 2	0 $\pm$ 0	< 0.0001	95	1231 $\pm$ 503	37 $\pm$ 60	< 0.0001	139	74 $\pm$ 27	3 $\pm$ 5	< 0.0001
52	0 $\pm$ 0	0 $\pm$ 0	< 0.0001	96	95 $\pm$ 39	3 $\pm$ 4	< 0.0001	140	2 $\pm$ 1	0 $\pm$ 0	< 0.0001
53	38 $\pm$ 15	1 $\pm$ 1	< 0.0001	97	8 $\pm$ 3	1 $\pm$ 1	< 0.0001	141	0 $\pm$ 0	0 $\pm$ 0	< 0.0001
54	2 $\pm$ 1	0 $\pm$ 0	< 0.0001	98	0 $\pm$ 0	0 $\pm$ 0	< 0.0001	142	0 $\pm$ 0	0 $\pm$ 0	< 0.0001
55	37 $\pm$ 13	4 $\pm$ 4	< 0.0001	99	1 $\pm$ 0	0 $\pm$ 0	< 0.0001	143	1 $\pm$ 0	0 $\pm$ 0	0.000
56	2 $\pm$ 1	0 $\pm$ 0	< 0.0001	100	0 $\pm$ 0	0 $\pm$ 0	< 0.0001	144	0 $\pm$ 0	0 $\pm$ 0	0.001
57	115 $\pm$ 44	7 $\pm$ 7	< 0.0001	101	1 $\pm$ 0	0 $\pm$ 0	< 0.0001	145	0 $\pm$ 0	0 $\pm$ 0	< 0.0001
58	5 $\pm$ 2	0 $\pm$ 0	< 0.0001	102	0 $\pm$ 0	0 $\pm$ 0	< 0.0001	146	0 $\pm$ 0	0 $\pm$ 0	0.000
59	53 $\pm$ 27	10 $\pm$ 10	< 0.0001	103	2 $\pm$ 1	0 $\pm$ 0	< 0.0001	147	1 $\pm$ 0	0 $\pm$ 0	0.001
60	2 $\pm$ 1	0 $\pm$ 0	< 0.0001	104	0 $\pm$ 0	0 $\pm$ 0	< 0.0001	148	0 $\pm$ 0	0 $\pm$ 0	0.004
61	350 $\pm$ 230	73 $\pm$ 73	0.005	105	4 $\pm$ 1	1 $\pm$ 1	< 0.0001	149	4 $\pm$ 1	6 $\pm$ 4	0.694
62	85 $\pm$ 5	2 $\pm$ 2	0.004	106	1 $\pm$ 0	0 $\pm$ 0	< 0.0001	150	1 $\pm$ 0	1 $\pm$ 1	0.934
63	21 $\pm$ 1	0 $\pm$ 0	0.000	107	34 $\pm$ 10	6 $\pm$ 8	< 0.0001	151	5 $\pm$ 1	1 $\pm$ 1	< 0.0001
64	0 $\pm$ 0	0 $\pm$ 0	0.002	108	7 $\pm$ 2	1 $\pm$ 1	< 0.0001	152	1 $\pm$ 0	0 $\pm$ 0	< 0.0001
65	6 $\pm$ 2	5 $\pm$ 5	0.275	109	30 $\pm$ 11	4 $\pm$ 5	< 0.0001	153	10 $\pm$ 11	1 $\pm$ 2	< 0.0001
66	4 $\pm$ 1	0 $\pm$ 0	< 0.0001	110	4 $\pm$ 1	0 $\pm$ 1	< 0.0001	154	2 $\pm$ 1	0 $\pm$ 0	< 0.0001
67	420 $\pm$ 157	13 $\pm$ 21	< 0.0001	111	5 $\pm$ 1	1 $\pm$ 1	< 0.0001	155	10 $\pm$ 3	1 $\pm$ 1	< 0.0001
68	24 $\pm$ 9	1 $\pm$ 1	< 0.0001	112	0 $\pm$ 0	0 $\pm$ 0	0.000	156	1 $\pm$ 0	0 $\pm$ 0	< 0.0001
69	148 $\pm$ 55	4 $\pm$ 7	< 0.0001	113	0 $\pm$ 0	0 $\pm$ 0	< 0.0001	157	0 $\pm$ 0	0 $\pm$ 0	0.005
70	8 $\pm$ 3	0 $\pm$ 0	< 0.0001	114	0 $\pm$ 0	0 $\pm$ 0	0.004	158	0 $\pm$ 0	0 $\pm$ 0	0.011
71	13 $\pm$ 4	1 $\pm$ 1	< 0.0001	115	1 $\pm$ 0	0 $\pm$ 0	< 0.0001	159	0 $\pm$ 0	0 $\pm$ 0	0.010
								160	0 $\pm$ 0	0 $\pm$ 0	0.004

<sup>a</sup> Zero values are rounded off concentrations of volatile organic compounds which were measured at ppt levels. Because of the variance between samples, decimals are not shown.

reference values. All resulting data sets were subjected to a combination of pre-processing (near-limit of detection (LOD)-thresholding, normalizing, log- or autoscaling, mean-centering) and OCC algorithms. The latter included k-Nearest Neighbour (kNN), One Class Support Vector Machines (OCSVM with radial kernel and grid-search parameter optimization), PCA with Q statistic measures (PCA/Q) and Soft Independent Modeling of Class Analogy (SIMCA) algorithms. Each model was fed with a 25  $\times$  repeated random leave-10-out cross-validation of the “normal” samples, all low-grade nutmeg samples were not used to build the model, their class distances were only predicted. Initial results were recorded as distance from class units, a posterior confidence limit on the baseline set's distance distribution was used as classification boundary. The top model was considered the model performing superior of each algorithm and all pre-processing method combinations. The performance was evaluated in terms of the best discrimination between the baseline (normal, quality nutmeg) samples from the low-grade nutmeg samples. Subsequently, based on this performance comparison, the best models of each of the four OCC algorithms were

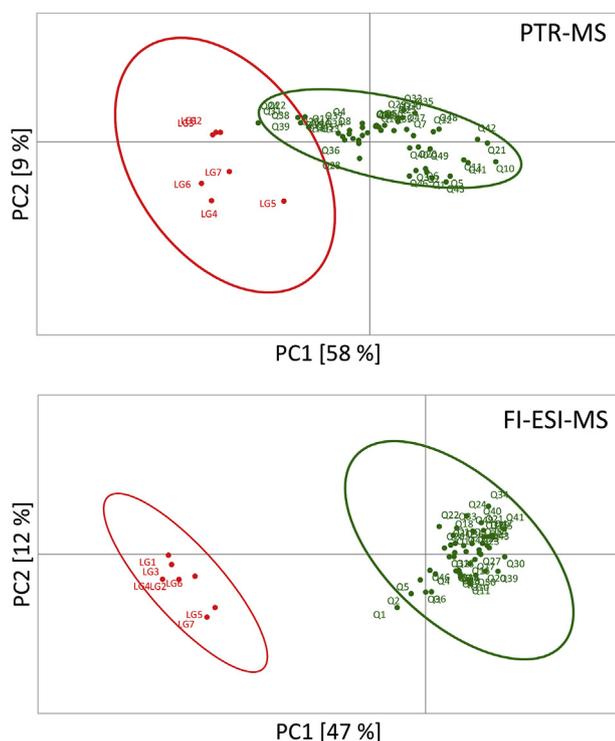
compared, and the model with superior performance - based on the results of the validation set - were selected. Finally, LODs of low-grade nutmeg mixed in normal nutmeg were estimated by generating 500 virtual mixtures of random good quality and low-grade samples at 10 different mixing ratio. These virtual samples were classified, and the LOD given is the concentration at which at least 95% of the samples were classified as deviating.

### 3. Results and discussion

#### 3.1. Characterization

##### 3.1.1. Gross composition of quality nutmeg

The total ash, acid insoluble ash, moisture and volatile oil contents of the quality spices showed a range of values, the frequency distributions of each parameter are presented in Fig. 1. Mean ash content amounted 2.8  $\pm$  0.5% w/w (range 1.9–4.2% w/w), the acid insoluble ash content (sand) 1.1  $\pm$  0.5% w/w (range 0.1–3.0% w/w), moisture:



**Fig. 3.** Plot of the first two dimensions of Principal Component Analysis of the PTR-MS and FI-ESI-MS fingerprints (no data pre-processing). Q codes and green colour indicate quality nutmeg samples and LG codes and red colour low-grade nutmeg samples. Circles present 95% confidence ellipses. One quality nutmeg sample with a high negative score on PC1 and high positive score on PC2 is located out of sight outside the plot area. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

10.2 ± 1.7% w/w (range 7.1–16.3% w/w), and volatile oil 7.7 ± 1.4 mL/100 g (range 4.6–12.0 mL/100 g). The ash contents of 14 of the 50 samples exceeded the specified/recommended maximum value of 3.0% w/w (ISO standard 6577:2002 (2002); ESA, 2015). The acid insoluble ash contents exceed the threshold of 0.5% w/w in 27 of the 50 samples. Hence, some sand was present in the samples, with one sample comprising as much as 3% sand. All samples that exceeded the ash content recommended value also exceeded the acid insoluble ash content value. Twenty of the 50 samples exceeded the 10% w/w moisture content maximum and 11 of the samples did not meet the minimum requirement for the volatile oil content (6.5 mL/100 g). There is no overlap between samples with a high moisture content and low volatile oil content, except for one sample. Hence, one is unlikely to cause the other. Six samples are the most divergent in the group, they are not meeting the set requirements for three out of the four variables. For clarity, samples do not have to meet these specifications unless demanded by the buyer. There is no legal basis, although this ISO standard and ESA recommendations help to set quality specifications.

Some studies have reported on the composition of nutmegs. For instance, fresh green nutmegs comprise approximately 40% moisture, 11% volatile oil, 30% starch, and 12% crude fibre (Krishnamoorthy & Rema, 2011). Fresh products comprise obviously more moisture than dried material, which also affect the relative concentrations of other components. However dried seeds consist of ca. 30–55% of oil, which can be divided in two types: “essential oil” that accounts for 5–15% of its seed weight and “fixed oil” which is also termed “nutmeg butter” that accounts for 24–40% (Farag, Mohsen, and El-Gendy (2018). Both products are extracted from the nutmeg material. The fixed oil is a semi-solid, reddish brown material with both the smell and taste of nutmeg. It comprises trimyristin (73%), essential oil (13%),

unsaponifiable constituents (9%), oleic acid (as glyceride) (3%), resinous materials (2%), linolenic acid (as glyceride) (0.5%). These are numbers for fixed oil, in which case the essential oil is not removed prior to extraction. After initial removal of the essential oil, the fixed oil comprises more trimyristin (84%), unsaponifiable constituents (10%), oleic acid (as glyceride) 4%, resinous material 2%, and linolenic acid (as glyceride) 0.6% (Krishnamoorthy & Rema, 2011; Marcelle, 1995).

### 3.1.2. Fingerprints of volatile compounds: PTR-MS

All samples were subjected to PTR-MS analysis. Rich profiles of volatile compounds were obtained: the mean fingerprints of all quality and the low-grade nutmeg samples are presented in Fig. 2. The low-grade nutmeg presents similar volatiles as the quality nutmeg, but generally in lower concentrations. The overall intensity (cumulated intensity of individual masses) of the low-grade nutmeg is about 10% of the intensity of the quality nutmeg. Concentrations of individual masses for quality and low-grade nutmeg are listed in Table 2, together with the significance of the differences between these two groups. The volatile compounds of nutmeg have been identified in a previous study of ours (Silvis et al., 2019). Predominant masses (mass-to-charge ratio) in the current study can be linked to particular compounds or fragments, for example methanol ( $m/z$  33), propenal ( $m/z$  57), acetic acid ( $m/z$  61), terpene fragments ( $m/z$  67,  $m/z$  81,  $m/z$  93,  $m/z$  95), furan/isoprene ( $m/z$  69), p-cymene ( $m/z$  135), and monoterpenes ( $m/z$  137) and their isotopes. These results are in agreement with Krishnamoorthy and Rema (2011) who reported on the volatile compounds in nutmeg oils of different provenance. They determined many terpenes, and sabinene (27%),  $\alpha$ -pinene (18%), myristicin (14%),  $\beta$ -pinene (10%), and terpinen-4-ol (7%) dominated in the oil of Indonesian nutmegs.

Many masses show significant differences between the two groups (Mann-Whitney test,  $p < 0.01$ ): 110 masses out of the 133 masses measured (83%). Only one mass ( $m/z$  31) presents a significantly higher value for the low-grade nutmeg, for all other masses the concentrations of the quality nutmeg are exceeding those of the low-grade counterparts. It is remarkable, however, that methanol ( $m/z$  33) and ethanol ( $m/z$  47) are more abundant in the low-grade nutmeg. Admittedly, the difference is not significant ( $p < 0.01$ ), which is due to the considerable variation in the low-grade samples. However, in the particular samples in which these compounds are present in large quantities, it points in the direction of use of solvents, presumably residue from processing (extraction). The larger variation in the low-grade nutmeg group is also reflected in the average value of the coefficients of variance of individual masses. This value amounts for the quality nutmeg group 44% and for the low-grade nutmeg group 117%. This may be due to the wide variety of spent materials and applied processing included in the low-grade sample group.

PCA on the PTR-MS fingerprint data reveals distinct clustering of both the quality nutmeg and the low-grade nutmeg samples (Fig. 3). Although various pre-processing methods were trialled, no data pre-processing was actually required for this first exploration since sufficiently separated groups were obtained without any pre-processing. Nearly all masses show high positive loadings in the first dimension, which are associated with the quality nutmeg samples. However, a few masses, among which are methanol and ethanol, present high negative loadings in the first dimension and are associated with low-grade nutmeg. Because of the distinct differences already seen in the univariate data analysis, the quality nutmeg and low-grade nutmeg sample groups are clearly distinguished. These distinct fingerprints are promising for further development of this method for authentication purposes.

Correlation of the cumulated mass intensities per sample as a measure for overall intensity and the compositional data (from section 3.1.1.) reveals that the overall intensity of volatiles and ash ( $r = -0.585$ ,  $p < 0.0001$ ), as well as the acid insoluble ash ( $r = -0.529$ ,  $p < 0.0001$ ) is significantly negatively correlated. Thus, the more sand or minerals are present, the lower the concentration of

**Table 3**  
Comparison of mass-to-charge ratio intensities of quality nutmeg and low-grade nutmeg measured by FI-ESI-MS (mean of all samples per group ± standard deviation), P-values indicate significance of differences (Mann-Whitney test).

Mass [m/z]	Quality nutmeg n = 50 [kcounts <sup>a</sup> ]	Low-grade nutmeg n = 7 [kcounts]	p-value	Mass [m/z]	Quality nutmeg n = 50 [kcounts]	Low-grade nutmeg n = 7 [kcounts]	p-value	Mass [m/z]	Quality nutmeg n = 50 [kcounts]	Low-grade nutmeg n = 7 [kcounts]	p-value
77.0383	28 ± 6	1 ± 3	< 0.0001	237.1483	3347 ± 1050	772 ± 756	< 0.0001	412.1802	2567 ± 744	341 ± 161	< 0.0001
79.0540	149 ± 32	2 ± 5	< 0.0001	238.1512	289 ± 79	72 ± 60	< 0.0001	413.1350	407 ± 137	443 ± 257	0.688
81.0697	1277 ± 258	47 ± 123	< 0.0001	239.1616	4856 ± 2145	695 ± 486	< 0.0001	415.2359	548 ± 147	273 ± 123	< 0.0001
82.0729	36 ± 8	1 ± 3	< 0.0001	241.0851	91 ± 101	734 ± 718	0.078	416.2397	106 ± 30	61 ± 10	< 0.0001
91.0540	1079 ± 217	5 ± 12	< 0.0001	245.1144	684 ± 395	22 ± 30	< 0.0001	425.1929	2456 ± 591	374 ± 183	< 0.0001
92.0592	43 ± 11	0 ± 0	< 0.0001	249.1472	48 ± 14	5 ± 13	< 0.0001	426.1961	477 ± 109	83 ± 40	< 0.0001
93.0696	297 ± 48	18 ± 47	< 0.0001	251.1632	153 ± 39	55 ± 50	< 0.0001	427.1718	2435 ± 751	319 ± 198	< 0.0001
94.0410	475 ± 100	0 ± 1	< 0.0001	253.1421	59 ± 17	17 ± 22	< 0.0001	428.1747	414 ± 148	45 ± 47	< 0.0001
102.0910	55 ± 11	174 ± 83	0.001	255.2313	68 ± 15	222 ± 39	< 0.0001	439.1715	1214 ± 2272	1 ± 3	< 0.0001
103.0540	1742 ± 380	11 ± 7	< 0.0001	256.2629	29 ± 20	86 ± 4	< 0.0001	441.1879	15571 ± 5259	2755 ± 1904	< 0.0001
105.0696	1429 ± 276	47 ± 36	< 0.0001	260.9588	43 ± 7	36 ± 5	0.025	442.1906	2811 ± 1257	452 ± 289	< 0.0001
107.0489	1872 ± 329	139 ± 24	< 0.0001	263.0673	1416 ± 1709	574 ± 528	0.234	443.2672	3167 ± 815	1498 ± 232	< 0.0001
111.1165	405 ± 134	32 ± 83	< 0.0001	265.1039	178 ± 49	50 ± 37	< 0.0001	444.2707	544 ± 129	265 ± 44	< 0.0001
115.0538	163 ± 33	1 ± 3	< 0.0001	267.2676	117 ± 34	30 ± 28	< 0.0001	445.1186	257 ± 262	130 ± 42	0.383
117.0694	110 ± 22	1 ± 3	< 0.0001	270.2785	48 ± 34	21 ± 2	0.087	449.2839	124 ± 65	122 ± 17	0.568
118.0409	671 ± 151	17 ± 7	< 0.0001	273.2569	80 ± 18	47 ± 14	< 0.0001	453.1875	18902 ± 3969	2083 ± 1179	< 0.0001
119.0487	271 ± 86	0 ± 1	< 0.0001	277.2152	25 ± 15	5 ± 13	0.001	454.1909	3794 ± 870	385 ± 201	< 0.0001
120.0564	117 ± 23	1 ± 1	< 0.0001	278.9693	63 ± 33	27 ± 33	0.002	455.1942	281 ± 95	6 ± 17	< 0.0001
121.0644	1172 ± 247	20 ± 19	< 0.0001	282.2785	62 ± 71	32 ± 5	0.055	457.1616	250 ± 134	468 ± 299	0.193
124.0514	157 ± 70	0 ± 1	< 0.0001	283.2625	72 ± 17	61 ± 7	0.031	459.1322	554 ± 133	26 ± 35	< 0.0001
125.0592	106 ± 25	62 ± 155	0.002	284.2943	29 ± 22	67 ± 2	0.000	460.1354	97 ± 60	1 ± 4	0.000
131.0013	4446 ± 270	3270 ± 528	< 0.0001	285.2417	173 ± 33	276 ± 85	0.001	461.3596	183 ± 104	3 ± 6	< 0.0001
132.0044	46 ± 6	30 ± 5	< 0.0001	289.2928	1973 ± 567	1163 ± 117	< 0.0001	467.4084	174 ± 79	164 ± 137	0.817
133.0644	2488 ± 451	72 ± 35	< 0.0001	293.0689	77 ± 41	4 ± 9	< 0.0001	469.1616	514 ± 110	591 ± 339	0.748
135.0800	1305 ± 285	29 ± 37	< 0.0001	299.1977	429 ± 119	192 ± 96	< 0.0001	473.1475	177 ± 82	8 ± 15	< 0.0001
137.0595	1310 ± 314	33 ± 31	< 0.0001	307.2250	50 ± 25	14 ± 11	< 0.0001	475.4138	102 ± 42	56 ± 6	< 0.0001
138.0671	153 ± 32	1 ± 1	< 0.0001	311.1266	25 ± 12	62 ± 30	< 0.0001	483.1983	262 ± 92	27 ± 38	< 0.0001
139.0386	131 ± 28	6 ± 16	< 0.0001	313.2732	416 ± 99	848 ± 119	< 0.0001	485.2652	120 ± 42	1 ± 2	< 0.0001
147.0502	1439 ± 143	557 ± 89	< 0.0001	314.2763	76 ± 17	127 ± 12	< 0.0001	495.4397	872 ± 464	1414 ± 586	0.017
148.0516	302 ± 51	35 ± 4	< 0.0001	316.3207	10726 ± 1863	6433 ± 267	< 0.0001	496.4434	256 ± 123	374 ± 137	0.019
150.0673	283 ± 151	15 ± 12	0.002	317.3239	1216 ± 425	739 ± 48	< 0.0001	503.1579	242 ± 95	24 ± 37	< 0.0001
151.0751	1614 ± 291	62 ± 46	< 0.0001	321.2093	84 ± 30	2 ± 5	< 0.0001	507.4012	50 ± 23	27 ± 21	0.010
152.0465	158 ± 40	0 ± 1	< 0.0001	323.1457	91 ± 85	18 ± 23	0.007	509.4555	65 ± 20	35 ± 28	0.007
154.0623	1998 ± 458	15 ± 12	< 0.0001	325.1429	2415 ± 495	1144 ± 621	< 0.0001	511.2544	84 ± 35	101 ± 15	0.063
155.0660	108 ± 18	11 ± 28	< 0.0001	327.1585	4974 ± 952	2129 ± 918	< 0.0001	515.2755	227 ± 59	16 ± 22	< 0.0001
161.0596	2222 ± 359	196 ± 113	< 0.0001	328.1620	637 ± 148	273 ± 100	< 0.0001	521.2523	1316 ± 374	254 ± 116	< 0.0001
163.0278	4687 ± 1665	6900 ± 760	0.000	329.1655	45 ± 13	16 ± 13	< 0.0001	523.4711	190 ± 99	268 ± 102	0.055
164.0785	257 ± 52	1 ± 4	< 0.0001	338.3413	2656 ± 2235	1998 ± 1033	0.953	524.4743	64 ± 36	89 ± 32	0.066
165.0910	3200 ± 587	637 ± 280	< 0.0001	339.3445	428 ± 323	300 ± 113	0.660	527.2195	63 ± 21	49 ± 8	0.011
166.0940	165 ± 58	41 ± 16	0.000	341.3043	3443 ± 1101	5568 ± 813	< 0.0001	533.2868	670 ± 165	39 ± 40	< 0.0001
171.0993	41 ± 17	746 ± 149	< 0.0001	342.3078	521 ± 155	726 ± 137	0.000	534.2900	200 ± 64	2 ± 5	< 0.0001
175.0750	89 ± 17	1 ± 2	< 0.0001	343.1531	121 ± 30	64 ± 38	0.000	535.4320	271 ± 88	305 ± 422	0.504
177.0908	1057 ± 221	10 ± 26	< 0.0001	347.1248	3108 ± 793	452 ± 207	< 0.0001	536.4356	86 ± 31	89 ± 117	0.429
179.0700	490 ± 124	9 ± 24	< 0.0001	348.1282	458 ± 109	74 ± 46	< 0.0001	547.4708	35 ± 44	55 ± 49	0.385
180.0777	264 ± 64	1 ± 3	< 0.0001	349.1404	8365 ± 1999	818 ± 310	< 0.0001	549.4865	270 ± 316	475 ± 431	0.743
181.0858	1945 ± 330	222 ± 218	0.008	350.1438	1113 ± 263	136 ± 44	< 0.0001	550.4900	93 ± 107	119 ± 127	0.467
182.0888	118 ± 21	11 ± 20	< 0.0001	351.1472	72 ± 23	5 ± 7	< 0.0001	551.5022	110 ± 97	221 ± 31	< 0.0001
184.0727	85 ± 59	14 ± 13	< 0.0001	353.2658	6681 ± 2362	6139 ± 905	0.730	552.5056	30 ± 39	80 ± 9	< 0.0001
184.9731	312 ± 37	1183 ± 59	< 0.0001	354.2691	847 ± 397	701 ± 116	0.107	553.4579	768 ± 473	265 ± 52	< 0.0001
186.0651	137 ± 84	0 ± 1	< 0.0001	355.1533	1571 ± 287	952 ± 566	0.040	554.4612	269 ± 147	95 ± 23	< 0.0001
189.0907	153 ± 48	4 ± 6	< 0.0001	357.1691	1703 ± 301	692 ± 317	< 0.0001	563.4637	83 ± 48	75 ± 31	0.374
191.0699	125 ± 33	10 ± 25	< 0.0001	359.1849	450 ± 280	1776 ± 574	< 0.0001	565.3750	48 ± 21	5 ± 5	< 0.0001
193.0860	19763 ± 3179	8946 ± 4477	< 0.0001	360.3195	187 ± 67	317 ± 63	0.000	569.4321	32 ± 24	77 ± 12	< 0.0001
194.0892	1385 ± 528	605 ± 381	0.000	363.0991	36 ± 19	76 ± 47	0.005	575.2969	65 ± 35	0 ± 1	< 0.0001
195.1016	21615 ± 3960	7860 ± 3008	< 0.0001	366.1174	16 ± 10	19 ± 19	0.456	579.5336	274 ± 90	608 ± 86	< 0.0001
196.1047	1788 ± 354	532 ± 268	< 0.0001	367.1513	1534 ± 323	195 ± 35	< 0.0001	580.5369	103 ± 32	222 ± 34	< 0.0001
197.1083	56 ± 15	12 ± 16	< 0.0001	368.1540	261 ± 82	17 ± 22	< 0.0001	589.4789	128 ± 54	119 ± 92	0.279
199.1688	134 ± 34	22 ± 58	0.000	369.1679	49 ± 31	401 ± 38	< 0.0001	590.4827	47 ± 20	39 ± 39	0.248
201.0905	233 ± 41	3 ± 9	< 0.0001	371.1844	1173 ± 314	903 ± 573	0.189	601.2762	137 ± 56	0 ± 0	< 0.0001
203.1064	1774 ± 307	116 ± 69	< 0.0001	377.1350	1935 ± 452	349 ± 148	< 0.0001	603.5336	38 ± 59	73 ± 69	0.591
205.1222	4262 ± 1188	87 ± 30	< 0.0001	378.1382	338 ± 102	8 ± 20	< 0.0001	609.3663	78 ± 45	0 ± 0	< 0.0001
207.1010	176 ± 52	175 ± 90	0.181	379.1511	1201 ± 262	174 ± 80	< 0.0001	615.1385	38 ± 14	42 ± 30	0.238
209.1170	11551 ± 1880	6621 ± 3100	0.000	380.1541	205 ± 84	17 ± 23	< 0.0001	619.5264	352 ± 207	459 ± 117	0.003
210.1203	875 ± 152	494 ± 284	0.003	381.2968	39017 ± 12267	31505 ± 3834	0.010	620.5294	136 ± 78	172 ± 39	0.005
211.0954	90 ± 14	8 ± 20	< 0.0001	382.3003	8053 ± 3004	6803 ± 1022	0.144	623.3335	47 ± 32	0 ± 0	0.000
215.0675	57 ± 20	0 ± 1	< 0.0001	383.3039	429 ± 210	350 ± 61	0.091	647.5576	1119 ± 744	861 ± 212	0.124
216.0756	2560 ± 535	1 ± 3	< 0.0001	385.2003	78 ± 22	18 ± 18	< 0.0001	648.5603	430 ± 249	328 ± 71	0.062
217.0830	359 ± 76	117 ± 79	< 0.0001	387.1793	86 ± 56	75 ± 50	0.807	649.5639	87 ± 46	65 ± 14	0.066
221.1167	129 ± 23	17 ± 22	< 0.0001	391.2832	109 ± 56	776 ± 86	< 0.0001	657.3772	107 ± 24	0 ± 0	< 0.0001
223.1329	9999 ± 2864	1486 ± 1336	< 0.0001	392.2867	15 ± 17	160 ± 19	< 0.0001	661.4060	298 ± 137	0 ± 0	< 0.0001
224.1359	799 ± 285	108 ± 82	< 0.0001	393.1662	581 ± 145	24 ± 55	< 0.0001	681.4703	40 ± 23	86 ± 43	0.002

(continued on next page)

Table 3 (continued)

Mass [m/z]	Quality nutmeg n = 50 [kcounts <sup>a</sup> ]	Low-grade nutmeg n = 7 [kcounts]	p-value	Mass [m/z]	Quality nutmeg n = 50 [kcounts]	Low-grade nutmeg n = 7 [kcounts]	p-value	Mass [m/z]	Quality nutmeg n = 50 [kcounts]	Low-grade nutmeg n = 7 [kcounts]	p-value
225.1117	1502 ± 643	994 ± 758	0.069	395.1455	1219 ± 668	109 ± 162	< 0.0001	703.4526	211 ± 114	223 ± 26	0.894
226.1149	123 ± 44	72 ± 43	0.018	397.1615	39144 ± 8337	4577 ± 2628	< 0.0001	717.5986	186 ± 72	180 ± 111	0.653
231.0987	1406 ± 1042	250 ± 342	< 0.0001	398.1652	8299 ± 1905	651 ± 386	< 0.0001	718.6020	79 ± 39	82 ± 53	0.874
233.1166	61 ± 41	33 ± 15	0.044	399.1681	440 ± 101	8 ± 20	< 0.0001	745.6297	382 ± 141	535 ± 184	0.031
235.1318	27 ± 8	25 ± 4	0.677	411.1770	15230 ± 3869	2109 ± 1157	< 0.0001	746.6335	150 ± 100	227 ± 120	0.042

<sup>a</sup> kcounts = counts \* 1000.

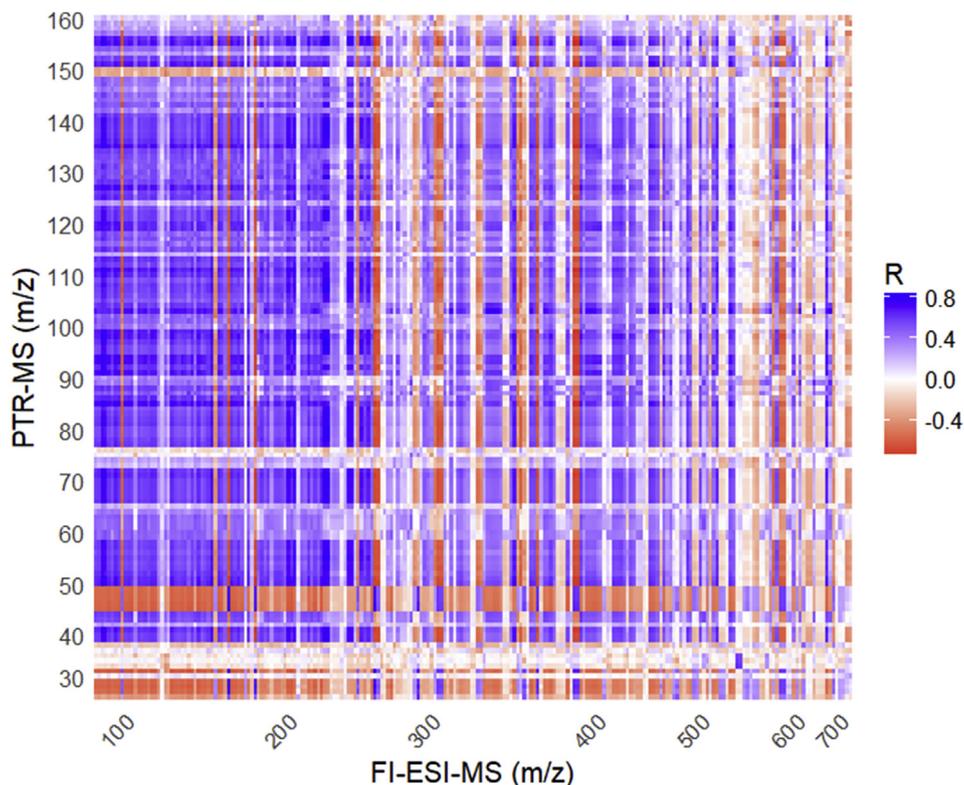


Fig. 4. Pearson's correlation coefficients for the correlation of PTR-MS and FI-ESI-MS intensity data.

Table 4

Best results of one-class classification models applied to the PTR-MS and FI-ESI-MS fingerprints of quality nutmeg and low-grade nutmeg. Numbers represent the number of correct predictions, followed by its percentage of correct predictions in brackets. A limit of detection (LOD) is estimated for each model/technique.

Technique	Algo-rithm <sup>a</sup>	Baseline set		Challenge set			Estimated LOD
		Quality nutmeg samples	Quality nutmeg pooled samples	Low-grade nutmeg samples	Mixtures of quality and low-grade nutmeg samples (1:1)		
PTR-MS	kNN	48 (96%)	5 (100%)	7 (100%)	35 (100%)	7%	
FI-ESI-MS	PCA/Q	48 (96%)	5 (100%)	7 (100%)	35 (100%)	30%	

<sup>a</sup> kNN = k Nearest Neighbour; PCA/Q = PCA with Q statistic measures.

volatile compounds measured. These components do not contribute to the nutmeg aroma, nor can they act as a solvent in the matrix. Therefore, their presence will only dilute/extend the nutmeg sample. On the contrary, the overall intensity of the volatiles and moisture content ( $r = 0.456$ ,  $p = 0.0004$ ) as well as volatile oil ( $r = 0.510$ ,  $p < 0.001$ ) are positively correlated. It is expected that a rise in volatile oil content results in a higher intensity of volatile compounds, but the strong correlation with the moisture content is more surprising. Although the moisture will dilute the sample, like sand, it can act as a solvent for the volatile compounds. Since most of them are of a non-polar nature, especially the terpenes, the gas/product partition coefficient (the concentration of a volatile in air divided by its concentration in the product

phase) will be considerably higher for these compounds with more moisture present in comparison to the partition coefficient for the same compounds in a fatty matrix even despite dilution of the initial concentration (van Ruth, de Vries, Geary, & Giannouli, 2002). Thus, the volatile compounds may be driven out by the water into the headspace due to a more favourable gas/product partition coefficient.

### 3.1.3. Fingerprints of non-volatile compounds: FI-ESI-MS

Samples were analysed by FI-ESI-MS and the fingerprints of the non-volatile compounds were acquired (Fig. 2). The quality nutmeg samples present an extensive fingerprint. On the contrary, a general reduction of the mass peak intensities is observed considering the low-grade

samples, but the decline is even more pronounced in the lower mass range, up to  $m/z$  300. The overall intensity (cumulated intensity of individual masses) of the low-grade nutmeg is about 40% of the intensity of the quality nutmeg. In this study, we use the mass spectra as a fingerprint without knowledge about the identity of the mass peaks. Therefore, we will just provide a few examples. Myristicin, for it is a psycho-active compound, may be of interest to look into. It has a molecular mass of 192, and is therefore, expected to become apparent at mass 193. This mass is presenting a high intensity, with significant higher values for quality nutmeg compared to low-grade nutmeg. Furthermore, mass 165 may reflect the presence of eugenol and isoeugenol, two compounds characteristic in essential oil of ripe nutmegs (Dupuy et al., 2013). Similarly mass 209 may be associated with elemicin, one of the abundant aromatic ether compounds in nutmeg oleoresin (Rodianawati, Hastuti, & Cahyanto, 2015). However, other analyses would be required to identify the compounds with greater certainty if one would like to use the data of this kind of analysis in a targeted manner. However, that was not the aim of this study.

Mass intensities of individual masses for the two nutmeg groups are listed in Table 3 with the significance of the differences. Of all the 228 masses, 175 (77%) present significant differences (Mann-Whitney tests,  $p < 0.01$ ) in intensities between quality nutmeg and low-grade nutmeg. For 150 of these masses the intensities are higher for the quality nutmeg group whereas 25 masses show higher intensities for the low-grade nutmeg group. On average, variation in the low-grade nutmeg groups is higher (CV% = 99%) than in the quality nutmeg group (CV% = 39%), which is a slightly lower than for the PTR-MS results.

PCA on the FI-ESI-MS data show very distinct clustering of quality nutmeg samples and low-grade nutmeg samples. One quality nutmeg sample is positioned further away from its counterparts, but it does not blend in with the low-grade sample group. Ageing or other factors may have determined this somewhat different fingerprint. It did not show an unusual PTR-MS fingerprint previously. Various pre-processing methods were examined, but in this case data were not pre-processed since this option resulted already in well-separated groups. The full separation of the two groups is once more promising for development of classification models.

Correlation of the cumulated mass intensities per sample as a measure for overall intensity and the compositional data (from section 3.1.1.) discloses that overall intensity of volatiles and ash ( $r = -0.055$ ,  $p = 0.687$ ) and acid insoluble ash ( $r = -0.110$ ,  $p = 0.942$ ), as well as moisture content ( $r = -0.137$ ,  $p = 0.310$ ) and volatile oil content ( $r = -0.076$ ,  $p = 0.574$ ) are not significantly correlated. Obviously, the features measured with FI-ESI-MS do not cover any of the aspects of the standardized methods. Dilution/extension with minerals/sand or moisture is not picked up with this method although it can distinguish the quality nutmeg and low-grade product fairly well.

### 3.1.4. Correlation of fingerprints of volatile and non-volatile compounds

The correlation between PTR-MS and FI-ESI-MS fingerprint data was examined with Pearson's correlation coefficients to understand the relationships between the concentration of volatile and non-volatile compounds. A plot representing the positive and negative correlations of masses of the two types of fingerprints presents an interesting tartan (Fig. 4). The plot demonstrates a considerable blue coloured area, which represents a positive correlation between intensities of volatile and non-volatile compounds. This means that when the concentrations of volatiles are high, so are the concentrations of the non-volatile compounds. This may relate to differences between samples with the same proportions of volatile and non-volatile compounds. The generally higher and lower concentrations may be due to natural variation, i.e. different qualities. Exceptions are some low molecular masses in PTR-MS analysis, which correlate negatively with the FI-ESI-MS data. These masses are primarily small molecules or fragments. This may be due to loss of volatiles over time in some samples resulting in lower low mass

volatile compounds with a constant proportion of non-volatile compounds. Furthermore, along the FI-ESI-MS axis, the blue colour representing high positive correlation with the volatile compounds is gradually fading and even changing into a negative correlation with increasing molecular weight of the non-volatile compounds that constitute the nutmeg matrix. Previously we noticed that the volatile compounds intensities were lower across the line for low-grade nutmeg compared to quality nutmeg. For the non-volatiles, that hold also for masses up to mass 300, but was less pronounced for higher molecular masses. This may be due to the removal of the more volatile compounds by extraction without changes in the higher molecular mass fraction, as is the case for the spent samples in the set. Considering the different phenomena observed in this correlation analysis, it is obvious that the two techniques provide complementing information.

### 3.2. Classification

The sample set for the classification consisted of the 50 quality nutmeg samples, the seven low-grade nutmeg samples, 5 pooled samples each consisting of ten quality nutmeg samples, and 35 mixtures combining each pooled sample and each low-grade nutmeg sample 1: 1. Pools and mixtures were also analysed by PTR-MS and FI-ESI-MS. Various algorithms and data pre-processing methods were evaluated for classification of the two groups, with the kNN algorithm (data pre-processing: normalisation, log-scaling and subsequent auto-scaling) working best for the PTR-MS data and the PCA/Q algorithm (data pre-processing: mean-centering) being most successful for the FI-ESI-MS data (see Table 4). Obviously, the perfect model would score 100% in all cases. If a model would fail to put quality nutmeg samples (baseline samples or pooled samples from the challenge study) into the quality nutmeg class, it generates false-positive results. Such a model used in routine applications would lead to false alarms, in the sense that quality nutmeg samples will be considered suspect, and someone who was selling good quality nutmeg would be unfairly accused of selling low-grade nutmeg. On the other hand, a method failing to detect low-grade samples generates false-negatives, which in practice could lead to non-conform products. Both problems should be avoided as much as possible.

Both approaches were very successful considering the correct classification rates. Forty-eight of the 50 baseline samples were assigned to the correct class, as well as all challenge samples including the pooled quality nutmeg samples, the low-grade nutmeg samples, and the 1:1 pooled quality nutmeg/low-grade nutmeg samples. Class distances for samples from the PTR-MS and FI-ESI-MS approaches were weakly correlated, which means that they seem to provide complementary information. None of the samples was consistently misclassified by both approaches. When considering the estimated LOD, PTR-MS (estimated LOD = 7% low-grade in quality nutmeg) was slightly ahead of the FI-ESI-MS approach (estimated LOD = 30% low-grade in quality nutmeg). Nevertheless, results of both approaches justify further database development. They will be a valuable addition to the current standardized nutmeg methods such as ISO standard 6577:2002 (2002), DNA-based methods to distinguish different botanical origins (Tallei & Kolondam, 2015), methods for irradiation detection in nutmeg (Chen et al., 2012), and methods for analyses of its volatile compounds in nutmeg fruits (Farag et al., 2018) or in its essential oils (e.g. Dupuy et al., 2013).

### 4. Conclusions and outlook

Both volatile and non-volatile fingerprints show distinct differences between quality nutmeg and low-grade nutmeg counterparts. Intensities of volatiles and non-volatiles show considerable positive correlation, but this effect diminishes and is even reversed with increasing molecular mass of the non-volatiles. Therefore, the two approaches complement each other and will pick up different quality and authenticity aspects. Both techniques allowed a nearly 100% correct

prediction of quality nutmeg and low-grade nutmeg samples, but the volatiles approach presented a slightly better LOD and requires less sample preparation. Strong fluctuations in the price of nutmeg lead to rapid changes in market dynamics and marketing opportunities, which may be addressed in an unfair way by those that override their moral compass. Current methodology may help to mitigate these risks. Further expansion of the database and subsequent use in practice will help to prevent us from being nutmegged in the future.

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