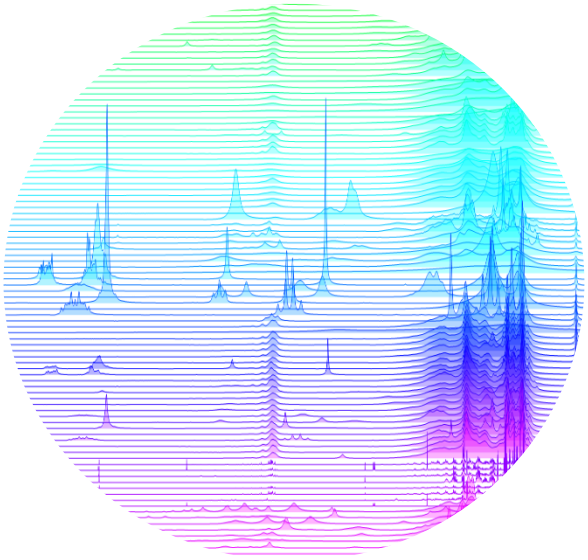
Is low-field NMR a complementary tool to GC-MS in quality control for essential oils?



Minor thesis

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July 2017

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Abstract

Adulteration of essential oils has a long history and is an everlasting issue for the essential oil industry. Demand for essential oils is expected to increase in the future. Quick and easy tools for quality control are required to cope with the production.

In this study 146 essential oils, most of them patchouli oils and several known adulterants were investigated by GC-MS and low-field 60 MHz NMR. Additionally the refractive index for a subset was determined. Chemometrics with ChemPattern was used for evaluation and showed that GC-MS is superior for pattern recognition which is crucial for quality control with a model quality of 97% and low number of false positives and negatives compared to NMR (86% model quality). Additionally it was possible to detect non-volatile adulterants with a semi-quantitative approach by GC. Furthermore a classification on high- and low-quality oils was possible based on the main constituents of the oil and a subsequent principle component analysis. The patchouli substitute Clearwood could also be distinguished by a visual inspection of the total ion current and the appearance of a possible ethylated patchoulol. NMR instead, demonstrated excellent distinction of genuine oils (no Clearwood) with Mahalanobis distance measure. Also here distinction between high and low patchoulol content could be achieved (high or low quality). A problem of peak broadening, which origin remains unresolved, could be solved by simple dilution. In contrast to that the traditional method of refractometry gave no satisfactory results and serve more as a complementary tool.

In conclusion we advise to further identify the problem of peak broadening to finalise the evaluation of NMR. Its future potential as a complementary tool in essential oil quality control seems promising.

Abbreviations:

PEO = Patchouli essential oil

GC-MS = Gas chromatography coupled with mass spectrometry

NMR = Nuclear magnetic resonance

QC = quality control

TIC = total ion current

RR_F = relative response factor

1 Introduction

Pogostemon cablin (Blanco) Benth. in the family of *Lamiaceae,* is a tropical plant with origins in the Philippines. Introduced in 1834 in India where it was mainly used as a repellent for insects, the plant soon came to Europe where French perfumers discovered the plant as a fragrance source (Murugan and Livingstone, 2010). Besides its medicinal use (Xian et al., 2011) and its repellent effect on certain insects (Zhu et al., 2003), the essential oil of *P. cablin* is nowadays still used as an important fragrance and base for the perfume and cosmetic industry. A strong earthy and woody character paired with its long-lasting properties makes it favourable for the industry. Essential oils in general are by definition the products of steam distillation or hydrodistillation from raw materials such as leaves (ISO9235 1997(E/F) 3.1).

According to data by Lawrence from 2007, patchouli oil belongs to the top 20 essential oils in terms of production with 1200 t annually. With more than 80% Indonesia produces the bulk of PEO's (Howarth et al., 2015; Lawrence, 2009). Market research up to 2024 predicts for the essential oil sector further growth as the demand for natural products is steadily increasing (Boren et al., 2015; grandviewresearch.com). Even though predictions are optimistic for the whole market, Howarth reports instability and unpredictability of the patchouli market with varying quality and price peaking seasons. Prices for one Kg of patchouli oil vary from 40 US $ up to 70 US $ for high quality oil (Market report Ultra International Spring 2015). An additional factor is the balance in supply and demand. Increasing demands cannot be covered by the producers. These conditions give space for low quality oils and deliberate adulteration of essential oils to supply to market demands.

Nowadays quality control is crucial to ensure constant quality and safety for the consumer. International standards like ISO should facilitate in identifying low quality oils by providing a guideline for a range of constituents for the respective oil. Especially for the essential oil of patchouli, the ISO-norms are contradictory to genuine PEO according to van Beek and Joulain. For instance the ISO norm allows a maximum concentration of 0.2% of α-gurjunene which is a compound found in gurjun balsam and can be classified as a marker for adulteration (van Beek and Joulain unpublished). The same issue was also mentioned by Boren et al. who indicated that certain standards are set by ISO and the Association Française de Normalisation (AFNOR) for some essential oils. However, there is still no agreement for the whole industry of essential oils which creates more space for low quality oils and questionable ingredients (Boren et al., 2015).

Molecular characterization and identification of the underlying processes will further clarify the term genuine as done by Burè and Sellier. They analysed patchouli oil and found several constituents typical for PEO and created with that report the first basis for qualitative and quantitative analysis of PEO (Buré and Sellier, 2004). Later van Beek and Joulain summarized data from over 100 PEO analysis which includes most of the data found by Burè and Sellier. Table 1.1 gives an overview over the main constituents of PEO.

Table 1.1: Main characterised constituents of PEO. Shown is the average concentration of the depicted constituents as well as its range which could be found in the number of studies. For further information see van Beek and Joulain. Taken from van Beek and Joulain (unpublished).

Constituent	Average %	Ranges %	Nr. of data
α-Pinene	0.09	0.01-0.3	28
β-Pinene	0.2	0.02-1	30
Limonene	0.03	0.01-0.3	22
δ-Elemene	0.52	0.01-1.9	19
β-Patchoulene	3.1	0.03-12	76
β-Elemene	0.88	0.18-1.9	39
(E)-β-Caryophyllene	3.3	0.75-20	77
α-Guaiene	11	2.9-23	62
Seychellene	6.4	2.3-13	65
α-Humulene	0.7	0.05-2	25
α-Patchoulene	4.4	1.2-13	68
Germacrene D	0.12	0.0-0.2	8
Aciphyllene	2.4	0.7-4.2	29
α-Bulnesene	14	2.9-23	80
Norpatchoulenol	0.93	0.11-4	37
Caryophyllene oxide	0.72	0.0-4.6	29
Pogostol	2.4	0.2-6.2	49
Patchoulol	39	11 to 72	101
Total without Pogostone	90.7	-	-
Pogostone	9.2	0.1-27.7	23
Total with Pogostone	99.9	-	-

The diversity of constituents in PEO which are mainly sesquiterpenes are formed by a small group of sesquiterpene synthases consisting of only five enzymes. Four of them use the common sesquiterpene-precursor farnesyl-diphosphate (FPP) as a substrate while the fifth catalyses a variety of reactions. This patchoulol-synthase is capable of forming at least 14 metabolites which are characteristic for PEO including patchoulol (Deguerry et al., 2006).

This research is important in terms of adulteration as recently Clearwood became available on the market. Clearwood is a PEO-like oil produced by the Brazilian Biotech company Amyris. With aid of the fundamental work of Deguerry the company implemented the metabolic pathway of patchouli synthesis in *Saccharomyces cerevisae* in a cheaper manner than extracting PEO from *Pogostemon cablin* (Daviet and Schalk, 2010). Although its produced by the same enzymes certain important compounds for the odour are missing like norpatchoulenol and nortetracyclopatchoulol. It is important to mention that geographic origin (Cornwell, 2010; Hu et al., 2006) or nutrient availability influence the composition of PEO as well (Singh and Ganesha Rao, 2009). These and other factors combined account for the great variability in the data as listed in Table 1.1.

Adulteration and falsification of products were already reported by Pliny the elder, a Roman academic who pointed this out in his book *"Naturalis Historia*" (Browne, 1909). He described various methods that were used back in time to detect fraud by odour, taste or colour. Knowledge about the exact properties of the testing compounds is crucial for quality control.

Pliny the elder summarised various methods which were developed to detect fraud and adulteration. The detection of balsam adulteration was tested as followed "[...] a drop of pure balsam, if placed in luke-warm water, will settle to the bottom of the vessel, whereas, if its adulterated it will float upon the surface like oil [...]" (Browne, 1909). Nowadays these tests are by far not enough to detect the sophisticated methods of adulteration but it shows nicely that this topic engaged people for more than 2000 years.

With the development of gas chromatography in the 50s of the last century, soon it became a powerful tool in investigating volatile compounds as shown for PEO (Hu et al., 2006). Nowadays it is an important tool for quality control in the sector of essential oils. By creating a representative chromatogram, different samples can be compared for their phytoequivalence. Even though geographic origin or cultivation practice may alter composition, the majority of constituents will remain the same. The advantages of GC-MS include high sensitivity and selectivity. Minor changes of composition due to oxidation or adulteration can be easily detected. However, preparation and running time are not to be neglected. Detected molecules can be easily identified with general mass spectral libraries although it is mentionable that general libraries are not well suited to identify essential oil compounds. Although the vast amount of volatiles can be detected without major effort, non-volatile or polar compounds are invisible for GC-MS (Liang et al., 2004). To overcome this obstacle a combination of different techniques like NMR could be aspired.

Over the last years low-field benchtop ^{1}H-NMR became an important tool for metabolomic fingerprinting and consequently for QC (Bluemich, 2016; Guthausen, 2016). Advantages over classical GC-MS is the high reproducibility in very short time on different machines as investigated by Keun et al. They could show that duplicated samples analysed on a 500 and a 600 MHz NMR in different laboratories almost produced the exact same spectra. Differences were explained by the machine variation itself and differences in pH adjustment in the two different laboratories (Keun et al., 2002). However, these results can also be translated to lower field strengths as the principle remains the same. Additionally to the time factor is the information content per measurement increased compared to GC-MS which discriminates for volatiles only, whereby NMR catches information of every molecule containing hydrogen. Nevertheless the information content in NMR depends highly on field strength and consequently determines the separation quality. Several studies could show already that high-resolution NMR is a suitable tool in QC for oil, juice, wine and beer (Dais and Hatzakis, 2013; Le Gall et al., 2001; Minoja and Napoli, 2014; Rodrigues and Gil, 2011).

High-field NMR was proven to work fine for quality control in the laboratory, but is it unsuitable for commercial QC in companies. High-field NMR requires highly skilled technical staff, has high maintenance costs and the costs for the machine itself are considerable. In recent years research and developer focused on low-field NMR with permanent magnets allowing small machines with low costs (magritek.com, oxford-instruments.com).

A study from Parker and colleagues showed that olive oil adulteration with hazelnut oil was possible to detect with 60 MHz bench-top spectrometer. Separation was achieved by qualitative means and signal differences in only one particular region of the NMR spectrum. Additionally mentioned was that due to the scarcity of information of 60 MHz spectra, the application of chemometrics was favoured to increase the information output of the sample batch. The limit of detection was evaluated with 13% w/w. Furthermore they concluded that FTIR, a frequently applied QC-tool, did not show any advantages over NMR (Parker et al., 2014).
Despite oil analysis, qualitative tests on meat were conducted and also here could the researcher show that by focusing on three single regions adulteration with horse meat could be certainly detected (Jakes et al., 2015). A quantitative approach with low-field NMR was tested by Pagès et al., who could show that with a reasonable long preparation and acquisition time (45 min) low-field and high-field NMR data yield the same accuracy up to a concentration of 2 mM. They concluded that low-field NMR is an excellent method for QC purposes (Pagès et al., 2014).

Aim of this thesis

It can be said that low-field NMR is slowly getting into quality control in several fields of the industry. The essential oil industry is still relying on GC-MS as its golden standard. To investigate a possible complementary use for 60 MHz NMR this study tried to tackle following points:

1) Create a reliable library of high quality patchouli oils with GC-MS
2) Investigate these oils with NMR and compare the data with GC-MS
3) Looking for approaches to separate genuine and adulterated samples which are suitable for quality control
4) Evaluate ChemPattern as chemometrics software

2 Materials and Methods

2.1 Database of oils

For this research 146 different oils and adulterants were analysed, comprising 96 patchouli oils from Hans Siwon† in Indonesia. 29 of these oils were not taken into account for the group of “genuine oils” for following reasons:

- Containing an unusual ratio of hydrocarbons to alcohols, like a low patchoulol or high Pogostone content (see Fig. 3.1 for common PEO pattern)
- Oils from other distilleries
- Containing suspicious peaks in NMR spectra which do not occur in genuine oil
- Adulterated samples
- NMR samples with a “blurry” spectrum which made comparison with clear spectra impossible.

A detailed list of the analysed and rejected oils can be found in the appendix (Table 5.1). The residual 67 PEOs were considered genuine based on GC-MS and NMR results

2.2 GC-preparation

~10 mg of accurately weighed essential oil samples were diluted in 1.00 mL of an internal standard solution of 0.250% (w/v) E,E-farnesol (627 µg) in 250 mL of methyl *tert*-butyl ether (MTBE).

Samples were analysed on a gas chromatograph from Agilent technologies 7890A equipped with the mass detector 5975C V MSD. Samples were injected with 7683B Series injector and 7683 Series autosampler. Used column was a HP-5MS 30 m x 0.25 mm x 0.25 µm.

The following GC-parameters were used:

Temperature profile	Rate [˚C/min]	Value ˚C	Hold [min]	Time [min]
Start		100	0	-
Ramp 1	3	175	0	25
Ramp 2	6	295	0	20
				Total: 45

Parameter	Value
Washes A [Acetone]	2
Washes B [Acetone]	2
Samples washes	2
Injection	1 µL
Split ratio	100 : 1
Inlet temperature	250 ˚C
Inlet heater	250 ˚C
Oven max.	300 ˚C
Total flow	24 mL/min
Septum purge flow	3 mL / min
Pressure [psi]	10.5
Column	constant flow
Solvent delay	3 min
scan parameter *m/z*	40 - 300

2.3 ^{1}H NMR preparation

NMR-tubes were first filled with approximately 25 µL tetramethylsilane (TMS) to correct for chemical shifts during analysis. Then, at least 600 µL of pure essential oil were added to the NMR-tubes. A solvent was avoided to keep the procedure as simple as possible.

Samples were run on a Spinsolve 60 carbon from Magritek (Magritek Aachen Germany). After the prescribed shim with 10% D_2O in 90% H_2O the following parameters were used:

Parameter	Value
Scans	32
Acquisition time	3.2 s
Repetition time	4 s
Pulse angle	30°

Scan number was set to 32 after trials with scans from 1 to 64. The noise was considered acceptable with 32 scans. Acquisition, repetition time and pulse angle were default values. Total running time was around 3 minutes.

To allow then an integration into ChemPattern, obtained spectra were loaded into MestReNova ("MNOVA", version 10, Mestrelab Research S.L., Santiago de Compostela Spain). Baseline correction was performed with the Magnitude function of the program as spectra were desired with a baseline as close to zero as possible. Manual phase correction was avoided as error-proneness is higher. Finally data were saved as "jcamp" file and imported into ChemPattern (Chemmind Technologies, Beijing Haidian China) for further analysis.

For the 600 MHz NMR PEO samples were run on a Bruker Avance III equipped with a cryoprobe. Data were adjusted and analysed with Topspin 3.5. Samples were prepared as 10% (w/v) of essential oil in $CDCl_3$. Pure compounds were measured at 1% (w/v) in $CDCl_3$. Tubes were filled with at least 600 µL.

Parameter	Value
Scans	8
Acquisition time [sec]	2.5
Delay [sec]	2
Pulse angle [°]	30
sweep width	22
TD (number of data points)	64000
Temperature [K]	300

2.4 Adulteration

For both GC and NMR analysis a high quality patchouli oil by Robertet in Grasse, France, supplied from Madagascar was used. Deliberate adulteration was performed with 20% (w/v) of an adulterant in 80% PEO from Robertet. Following adulterants were used (van Beek and Joulain unpublished):

Adulterant	Retailer	Product details
amyris oil	Naarden International	
benzyl alcohol	VWR International B.V.	
benzyl benzoate	Merck KGaA	
cedar wood oil	F.E.S Rotterdam	*Juniperus virginiana*
Clearwood	Givaudan	
copaiva balsam	anthemis.nl	E1070 *Coipafera officinalis*
diethyl phthalate	Sigma-Aldrich	
dioctyl phthalate	Sigma-Aldrich	
gurjun balsam	anthemis.nl	E3150 *Dipterocarpus alatus*
Hercolyn	Robertet - Grasse, France	
isobornyl acetate	Robertet - Grasse, France	
methyl benzoate	Sigma-Aldrich	
paraffin	Apotheek de Linge in Opheusden	
paraffin viscid	Sigma-Aldrich	
pepper oil	anthemis.nl	E1650 P*iper nigrum*
propyleneglycol	Apotheek de Bongerd in Ochten	
ricinus oil	anthemis.nl	E6150
vetiver oil	unknown	Origin: Haiti
1R - (-) myrtenol	Sigma-Aldrich	

As we expected that commercial patchouli oils are adulterated as well, 13 commercially available oils were purchased and analysed.

Retailer	Product details	Origin
Anthemis.nl	Patchouli 10 mL E9630	Malaysia
Anthemis.nl	Patchouli 5 mL E1630	Indonesia
Carl Roth		
Chi International B.V. Breda NL		
De Tuinen B.V.		
F.E.S. Rotterdam		
Jacob Hooy en Co (old)		
Jacob Hooy & Co B.V. (new)		
Keypharm	*Pogostemon cablin* from Physalis	Indonesia
Ladrome laboratoire		France
Naproz, Gezond & Wel		
Primavera life GMBH		
Sigma Aldrich	Pcode:1018477478	

2.5 Refractometry

The refractive index of the essential oils was measured on an Abbe refractometer ATAGO 1T Refractometer equipped with a ATAGO digital thermometer. The temperature during measurements was 23 °C. The wavelength for analysis was the D line with 589 nm.

2.6 Metal extraction

To investigate the role of metal ions in patchouli oil and their influence on the NMR spectra quality, an extraction with EDTA was conducted. ~1 g of PEO was dissolved in 10 mL of MTBE. This solution was three times extracted (3 x 5 mL) with a solution of 0.1 M EDTA + 0.05 M TRIS pH 8 in a separatory funnel. The oil solution was dried over $NaSO_4$. Finally the solution was filtered with a paper filter and the MTBE was evaporated with a Rotavap at 40 °C and 310 mbar. Residuals were dried with N_2. The oil was then prepared as described in §2.3.

2.7 Data analysis with ChemPattern

For data analysis the software ChemPattern Edition V2.0 from the company Chemmind technologies was used (Chemmind Technologies, Beijing Haidian). Obtained data were grouped into the following groups:

Group
Genuine 1
Genuine 2
Not representative
Commercial genuine
Commercial adulterated
Adulterants
Deliberate adulteration
Other species

Despite the 29 oils which were grouped as not representative (see 2.1 for elaboration) the remaining 59 genuine PEO were split into two randomized groups (Genuine 1 and 2).

2.7.1 GC-data TIC analysis

Peak identification and area were calculated by ChemPattern with the following parameters and applied to all samples:

Parameter	Value
Slope	100
Minimal peak width	0.1
Minimal height	0
Minimal height (%)	0.1
Minimal area	0
Minimal area (%)	0.1
Integration start	3 min
Integration stop	40 min

To correct for offsets in retention time peak alignment for assigned peaks was necessary. Peaks for PEO were assigned according to van Beek and Joulain (van Beek and Joulain unpublished). Peaks from adulterants were first analysed in pure from and compared to those of the 20% deliberate adulterated samples. Peaks from adulterants which were still visible at the 20% level were marked and labelled according to the adulterant. Table 6.2 gives an overview of identified peaks, their retention time and most important ions.

2.7.2 Semi-quantification

To detect “invisible” adulterations for GC-MS like paraffin which are non-volatile, total area comparisons of relevant patchouli peaks were conducted. Whereby the total area should be smaller in adulterated oils compared to genuine oils assuming 100% of the oil is volatile. Non-PEO peaks were subtracted for the total area calculations also when co-elution appeared. Certain compounds co-eluted with PEO compounds. An example is the co-elution of cycloseychellene and a copaiva balsam compound. Genuine PEO had a cycloseychellene area of around 0.5% while adulterated with copaiva balsam exceeded this area of 0.5% by far and was subsequently excluded.

For the analysis a triplicate of the reference oil from Madagascar (PEO from Robertet) was analysed on GC and the relative response factor (RR_F) was calculated:

$$RR_F = \frac{A_x}{A_i} * \frac{W_i}{W_x} \qquad [1]$$

A_x = area of all patchouli related peaks (minus internal standard)

A_i = area of internal standard (E,E-farnesol)

W_i = weight of internal standard in mg

W_x = oil weight in mg

With the average of the three response factors the weight of all patchouli related peaks in different oils was calculated:

$$W_x = \frac{A_x}{A_i} * \frac{1}{RR_F} * W_i \qquad [2]$$

2.7.3 Cosine similarity analysis

Conducted similarity analysis was based on cosine similarity. Cosine similarity transforms obtained data into a vector and compares then the angle between two vectors e.g., two TIC’s (chemmind.com/wiki).

Cosine similarity is defined as:

$$\cos(d_1, d_2) = \frac{d_1 * d_2}{||d_1|| * ||d_2||} \qquad [3]$$

Whereby d is the vectorised sample data

An example with random numbers to illustrate cosine similarity:

Sample 1 as vectorised data = (5, 0, 3, 0, 2, [...], n_x)

Sample 2 as vectorised data = (3, 0, 2, 0, 1, [...], n_y)

1) $$d_1 * d_2 = 5 * 3 + 0 * 0 + 3 * 2 + 0 * 0 + 2 * 1 + n_x * n_y = 25$$

2) $$||d_1|| = \sqrt{5 * 5 + 0 * 0 + 3 * 3 + 0 * 0 + 2 * 2 + n_x * n_x} = 6.48$$

3) $$||d_2|| = \sqrt{3 * 3 + 0 * 0 + 2 * 2 + 0 * 0 + 1 * 1 + n_y * n_y} = 4.12$$

4)

$$\cos(d_1, d_2) = \frac{d_1 * d_2}{||d_1|| * ||d_2||} = \frac{25}{6.48 * 4.12} = 0.96$$

Calculated cosine value	Meaning for cosine analysis
X = 1	Two vectors (TIC's) are identical
1 < X > 0	Two vectors are similar
X = 0	Two vectors are in 90˚ to each other

2.7.4 Mahalanobis distance measure

Similarity analyses for NMR was based on Mahalanobis distance. The Mahalanobis distance measure performs the following things:

- It transforms variables into uncorrelated variables
- It equals their variance to 1
- It calculates then Euclidean distance

$$D^2 = (x - m)^T * C^{-1} * (x - m)$$

D^2 = Mahalanobis distance

x = vector of data

m = vector of mean values of independent variables

C^{-1} = Inverse covariance matrix of independent variables

2.7.5 Principal component analysis

For multivariable analysis with more than three variables (dimensions) principle component analysis (PCA) from ChemPattern was used. Its aim is to transform the data into a linear relationship while preserving their most influencable variables. These new variables are called principle components (PC) 1, 2, 3 and so on. They are sorted by their influence on the variance on the dataset with PC1 = explains most variance in the data set (chemmind.com/wiki/).

2.7.6 Pattern recognition with k-nearest neighbour and model cross validation

K-nearest neighbour is a method to classify at least two unknown test datasets to their most proximal neighbour and assign them to a known training data set. The number of neighbours ranges from 1 to n. It is noteworthy that always an odd number should be preferred to avoid ties.

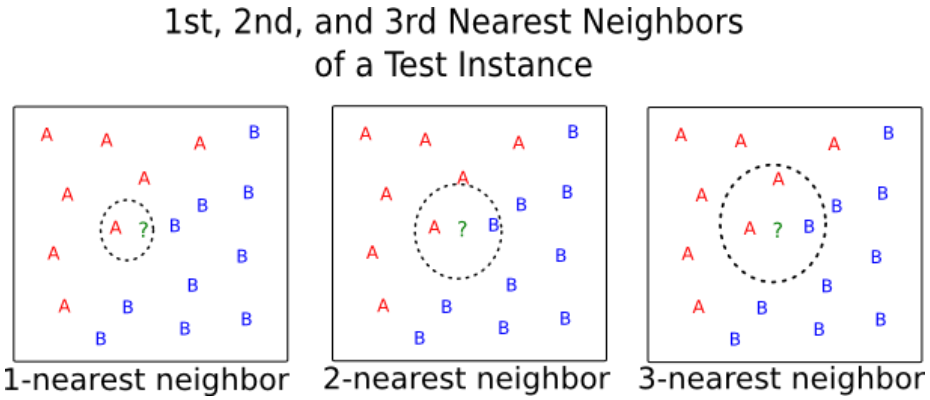


Figure 2.1: Shown is the theory behind k-nearest neighbour. Picture one illustrates when k=1, picture 2 when k=2 and picture 3 when k=3. Taken from: http://trevorwhitney.com/images/knn.png 08.06.2017

K-fold cross validation is used to validate an existing model with the underlying dataset without analysing new samples. Theory behind this is that the dataset is split into a subset consisting of a training set and a test set. Training set is used to build the model while the test set should validate its predictions. The dataset splitting was performed with the k-fold cross validation. Here the test set was split into seven folds (k=7). Now each fold predicts the training set. The aim of k-fold cross validation is to estimate expected predictions error. Also to aid in selecting the best fit model and not to overfit the model (D.L. Massart, 1997).

3 Results and Discussion

To generate a common pattern for GC as well as NMR, 73 patchouli oils from Indonesia and one patchouli oil from Madagascar were taken into account (Fig 3.1). Peak assignment for adulterants was conducted as described in materials and methods. Table 6.2 gives an overview of important peaks for GC-MS.

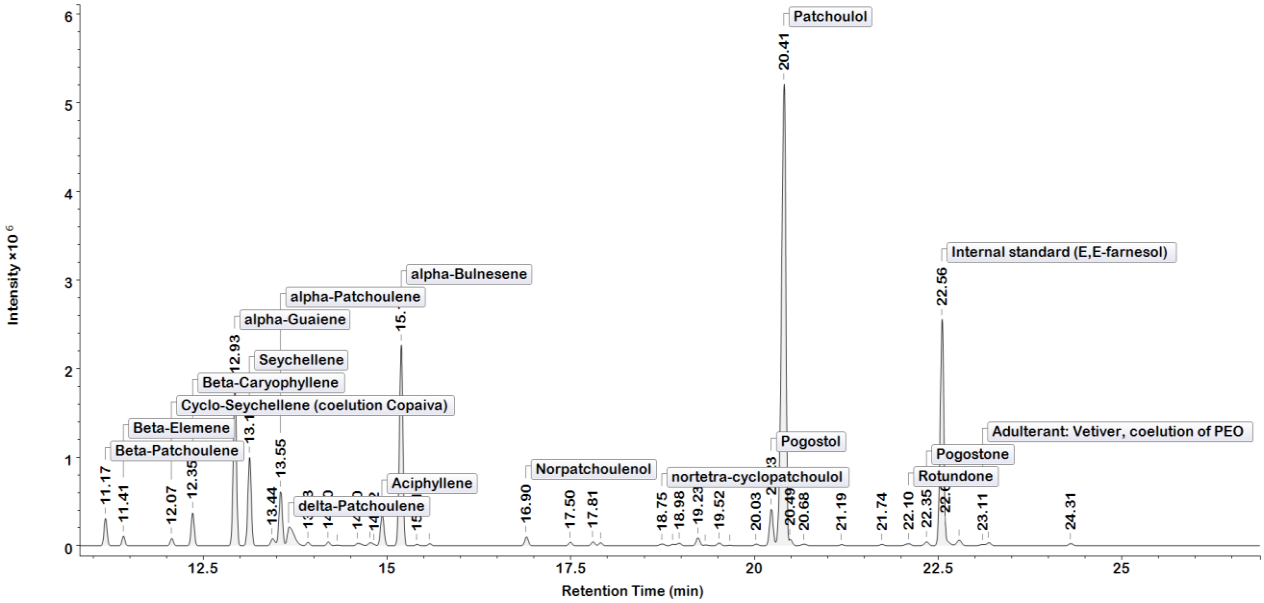


Figure 3.1: Fingerprint region by a combination of 73 different patchouli essential oils including genuine oils from Indonesia and commercial genuine samples. All constituents elute in the depicted time frame from 10 – 25 min. For all representative oils in this sample see appendix table 5.1 including PEO from Robertet. Note that cycloseychellene can co-elute with a constituent of copaiva balsam (see also Fig. 6.1). Same accounts for the peak at 23.11 were a vetiver-compound also co-elutes.

3.1 Semi-quantification

With properly assigned peaks from genuine PEO as well as deliberately adulterated samples, calculations on the basis that 100% of the genuine oil is volatile could be conducted to account for additional peaks or missing area percentages which can lead to adulteration. First the RR_F of a triplicate of PEO-Robertet was calculated to account for variations. Then with the average RR_F, the residual samples were calculated as described in materials and methods (2.6.2). Results are shown in table 3.1. Commercial samples can be clearly distinguished into two groups with five sample containing less than 90% of PEO related peaks while 8 samples showed high similarity to PEO-Robertet. Low patchoulol content was characteristic and several adulterants like benzyl alcohol, cedar wood or copaiva balsam were added to the adulterated commercial samples which were mainly old samples (from 1990's) or from an online aromatherapy shop (see Fig. 6.1). Commercial genuine samples like Physalis from Keypharm showed PEO content of 99% which indicates that nowadays commercial available PEO's from companies can be considered as genuine (especially observable with Jacob Hooy old vs. new).

Table 3.1: Percentage of genuine PEO in samples based on the average RR_F of a triplicate of PEO-Robertet. It was assumed that 100% of the injected oil is volatile. W_X is the calculated weight based on an average RR_F from three samples of PEO-Robertet. Green indicates that high similarity to genuine PEO exists 90% < x > 110%, while red indicates that samples should be treated suspiciously and non-genuine / low quality.

Samples	W_X [mg]	Original weight [mg]	% resemblance to genuine PEO
Genuine 1			
18	9.619	9.9	97.2%
37	9.338	10.0	93.4%
63	9.968	10.0	99.7%
Genuine 2			
16	11.104	10.0	111.0%
59	10.024	9.8	102.3%
70	10.580	10.5	100.8%
Commercial samples			
anthemis.nl Indonesia	7.270	9.8	74.2%
anthemis.nl Malaysia	2.894	9.7	29.8%
Carl Roth	5.860	10.1	58.0%
Chi International B.V Breda NL	9.338	9.9	94.3%
De Tuinen B.V.	9.349	10.1	92.6%
F.E.S Rotterdam	3.165	10.0	31.6%
Jacob Hooy en Co (old)	2.901	10.0	29.0%
Jacob Hooy (new)	9.623	10.0	96.2%
Ladrome laboratoire	9.448	9.7	97.4%
Naproz, Gezond & Wel	9.894	10.1	98.0%
Physialis PEO from Keypharm	9.798	9.9	99.0%
Primavera life GMBH	9.660	10.0	96.6%
Sigma Aldrich	9.970	10.1	98.7%
Deliberately adulterated			
20% benzyl benzoate	7.780	9.7	80.2%
20% paraffin	7.884	10.0	78.8%
20% Clearwood	10.294	10.0	102.9%
20% vetiver	8.040	9.8	82.0%
20% copaiva balsam	8.430	10.2	82.6%
20% gurjun balsam	7.906	10.0	79.1%
Not representative			
8	9.063	10.2	88.9%
45	11.278	10.4	108.4%
64	7.537	10.3	73.2%

It is noteworthy that most commercial samples contained α-copaene which should be treated as an indicator for adulteration at a level of >0.5% according to van Beek and Joulain. Indeed, all adulterated commercial samples showed higher level of α-copaene compared to genuine commercial samples which were all below the threshold of 0.5%. This emphasizes the argument that α-copaene can serve as a marker for adulteration at a threshold of 0.5%.

The deliberately adulterated samples on PEO-Robertet basis showed that the 20% adulteration could be certainly detected (Table 3.1). As expected Clearwood adulteration was impossible to distinguish from genuine samples. Analysed pure Clearwood is characterised by a lack of norpatchoulenol, nortetracyclopatchoulol and pogostone, the two first are very potent odour compounds of PEO (Spreitzer, 1992). Besides this, Clearwood contains a very unique molecule with a mass of 250 as a by-product during the production with *Saccharomyces cerevisiae* and is a clear indicator for Clearwood adulteration. The hypothesis is that an ethylation (CH_3-CH_2-R, MW patchoulol 222 + 28 = 250) of patchoulol takes place as alcohol is used during the production. (Fig. 6.2 and 6.3). Norpatchoulenol and nortetracyclopatchoulol are always present to a certain amount and can consequently not be used as an adulteration marker. Same holds for pogostone which may or may not be present in a PEO due to natural variation. The amount of identified by-product is not enough to distinguish the sample semi-quantitatively.

The genuine samples from the two different groups showed PEO content of more than 90% up to 110%. Natural variation and a different ratio of hydrocarbon to alcohol can be considered as the main reason for these values. Less than 10% adulteration does not seem feasible as natural variation is too high. For the non-representative samples it is shown how an odd ratio of hydrocarbons to alcohol influences the results. Sample 8 originates from another local distillery and has a low content of patchoulol while sample 64 contains a considerable amount of pogostone.

Sample 45 derives from the first 10 minutes of the distil process for PEO and has an unusual high amount of hydrocarbons while the important patchoulol is extracted later during the distillation and consequently lacks to a substantial amount in this sample. The similarity remains in the range and indicates a good oil. In summary this way of analysing the oil shows that adulteration is easy to detect if a common pattern exists and peaks from adulterants are known and can be assigned. However, for odd ratio of hydrocarbons to alcohol to further determine the quality of the oil, this method is not suitable.

3.2 Similarity analysis for GC

The previous approach of semi-quantification relied on manual selection of peaks and areas in Excel. With ChemPattern it is possible to immediately assign samples based on their TIC similarity. A representative pattern of “Genuine 1” served as a template for the software to compare any other sample with that. Default settings for cosine analysis in ChemPattern were chosen and no weighing for peaks was applied. Figure 3.2 shows that the common pattern generated with Genuine 1 could identify Genuine 2 as closely related and genuine. Commercially genuine samples were proximate as well. Small deviations can be explained by their overall smaller content of patchoulol compared to the high quality oils. While semi-quantification (see above) could easily distinguish samples which were adulterated with non-volatile compounds like paraffin (80% PEO area recovery instead of 100%) cosine similarity does not yield good results. As no additional peaks are in the chromatogram the software treats the sample similar and only accounts for the overall smaller amount for constituents. Surprisingly vetiver adulteration is also not detectable even though vetiver has a rich spectrum containing many constituents. Because weighing was not applied and vetiver itself does not show any remarkable peak area in adulterated samples, means, a very specific peak with at least 10% of the total area, similarity remains high. Implementing a threshold for similarity at 97.5% including the commercial genuine samples, adulteration with paraffin, vetiver, ricinus and Clearwood would not be distinguishable from genuine samples. By combining both approaches of semi-quantification and cosine similarity analysis it is possible to distinguish all adulterated samples except for Clearwood from genuine ones. Also is it possible to make predictions for quality in terms of patchoulol content as one of the main distinguishing features. If compared to the rest of genuine set are samples 10, 39, 4 and pat-ts 122 lst sh including the commercial genuine ones lower in patchoulol content than the rest of the samples which are higher in the similarity ranking (Fig. 3.2).

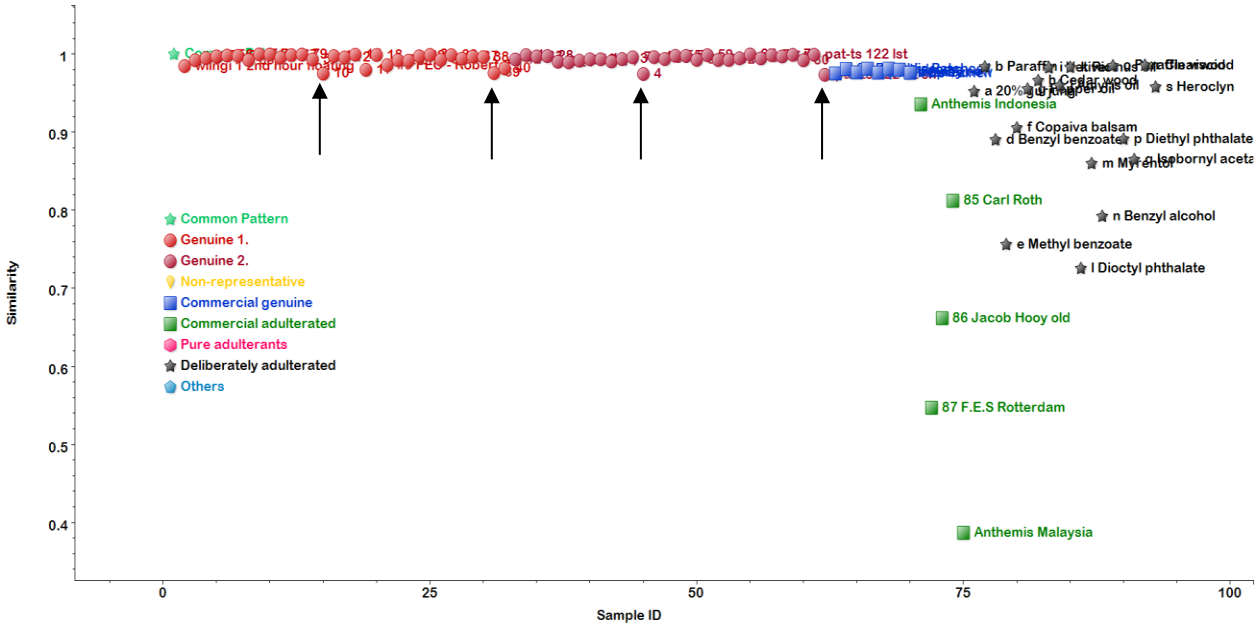


Figure 3.2: Cosine similarity analysis with ChemPattern for GC spectra. Group Genuine 1 served as common pattern and was assigned as “representative samples”. Remaining samples were assigned as “normal sample”. No weighing was applied. Indicated with arrows are samples 10, 39 and 4 and pat-ts 122 lst sh to indicate low patchoulol content.

If the commercial genuine samples would be included in the common pattern previous distinguished samples would be within the common pattern (Fig. 3.3). Patchoulol content as main distinguishing factor is gone. Those deliberately adulterated samples with less prominent peaks in the TIC like vetiver cannot be distinguished anymore.

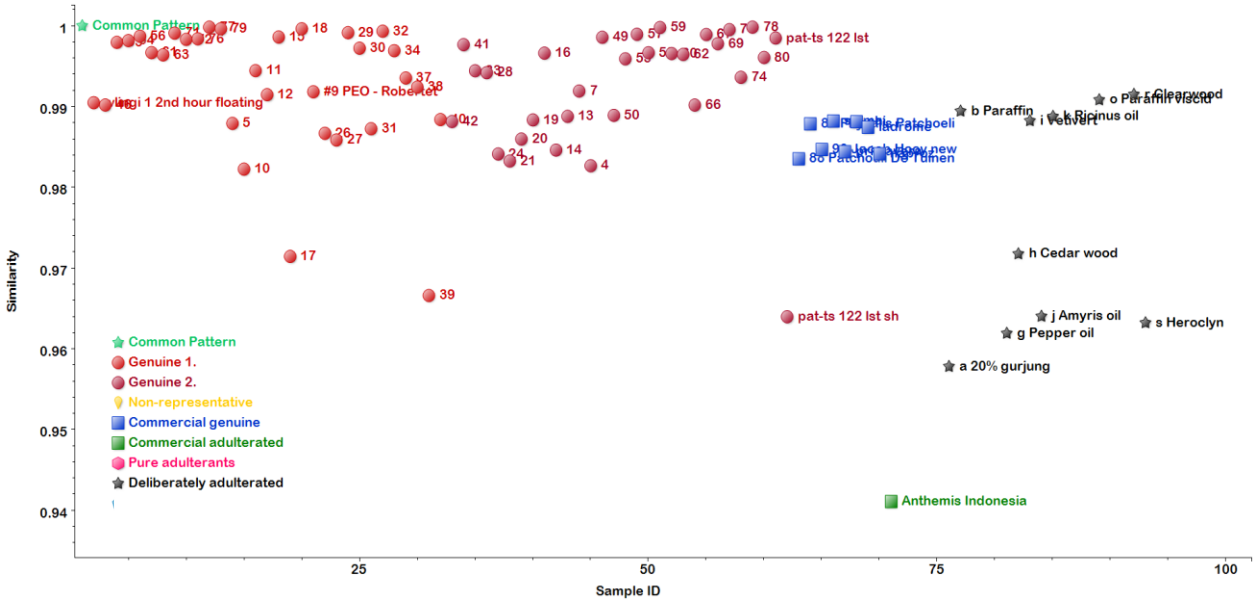


Figure 3.3: Cosine similarity analysis with ChemPattern for GC spectra. Group Genuine 1 and commercial genuine served as common pattern and was assigned as “representative samples”. Remaining samples were assigned as “normal sample”. No weighing was applied. Shown is a zoom to indicate which samples can’t be distinguished anymore.

3.3 Multivariate analysis for GC

As nature of adulteration is unknown in practise, a weighing factor for adulterants was neglected in favour of inherent constituents from PEO. ChemPattern is not providing so far a tool to distinguish on a yes or no basis any additional peak means, as soon as an additional peak appears in a spectrum, this oil would be assigned as an outlier oil. A similar approach was to weigh adulterants to 100% to achieve this. However, it did not seem practical as the distinction was based on adulterants only. Any new unknown adulterant would be undetectable as the peak is unknown. So the focus was on the characteristics of PEO itself. After assigning weights with aid of the biplot function of ChemPattern to the major contributors of variation, a clear distinction of genuine samples from adulterated samples was possible (Fig. 3.4). Principle component 1 explains the variation caused by patchoulol, α-guaiene, α-bulnesene and seychellene whereupon principle component 2 is an indicator for α-bulnesene, α-guaiene and seychellene only and consequently for hydrocarbons. A low patchoulol content is the first distinctive feature for adulterated samples. Even though the deliberate adulterated samples have a high quality oil as background, their lower level is an important indicator. Further on is the ratio of hydrocarbons to patchoulol of importance. High patchoulol oils have naturally less hydrocarbons and *vice versa*. In these experimental conditions co-elution was observed with certain compounds (gurjun and copaiva compounds with cycloseychellene).

These co-elutions cause an odd ratio and is subsequently an indicator of adulteration. By dividing samples with this kind of weighing, Clearwood adulteration remains invisible as Clearwood contains all necessary compounds which are used in this analysis. A possible distinction based on pogostone is not advised. Even though Clearwood contains no pogostone as the required enzymes are missing for that process but the natural level of pogostone is too variable to enable distinction on that constituent (see also Table 1.1). Summarized are low quality PEOs found in the lower left corner and are indicated by low patchoulol content or an odd ratio of hydrocarbons to patchoulol (which is mostly caused by low patchoulol content). Clearwood remains only detectable with a visual inspection of the TIC as the additional compound is minor.

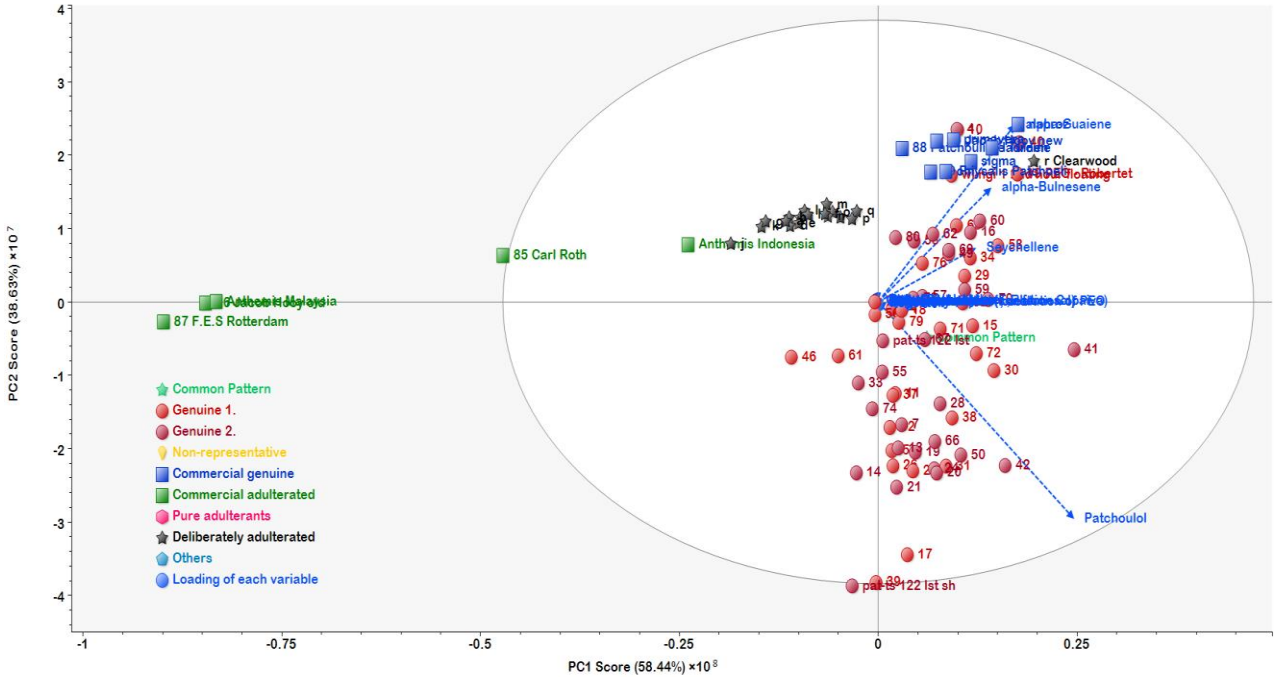


Figure 3.4: Biplot of Principle component analysis with ChemPattern for GC data. 89 independent variables (peaks) are taken into account. Group Genuine 1 was assigned as representative samples Four constituents are assigned with a weight: patchoulol = 15, α-bulnesene = 20, α-guaiene = 30, seychellene = 40. Choice was based on high values of Eigenvalues and their explanatory power when no weights were assigned.
Abbreviations for deliberately adulterated samples: a) gurjun b) paraffin d) benzyl benzoate e) methyl benzoate f) copaiva balsam g) pepper oil h) cedarwood i) vetiver j) amyris oil k) ricinus oil l) dioctyl phthalate m) myrtenol n) benzyl alcohol o) paraffin viscid p) diethyl phthalate q) isobornyl acetate s) Hercolyn.

3.4 Pattern recognition for GC

For quality control in practise a quick decision making tool is necessary. For that purpose the pattern recognition function of ChemPattern was chosen to assign samples quickly to a known training set based on a model created by these training sets. The results are shown in Figure 3.5.

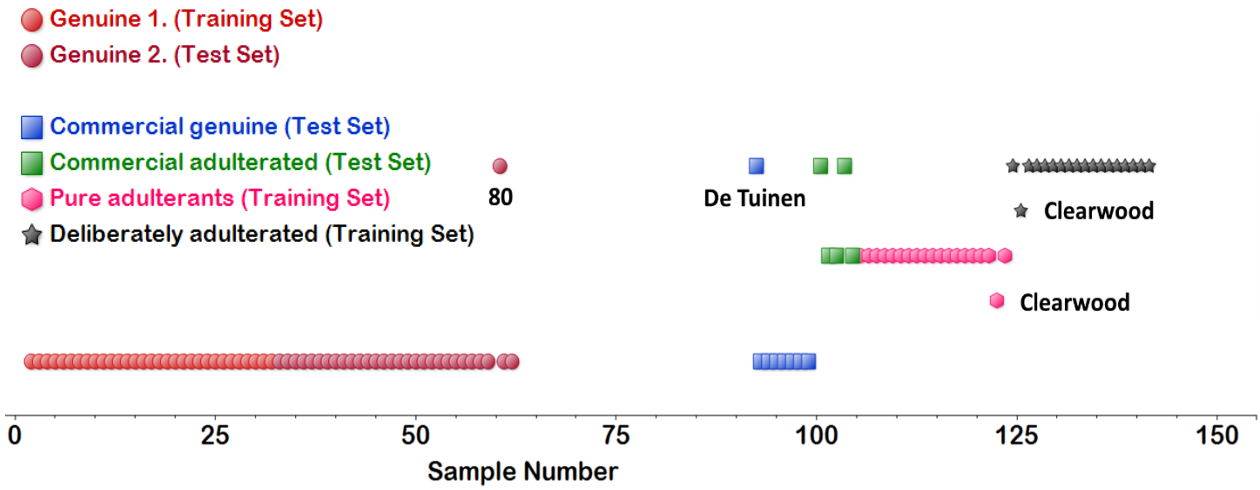


Figure 3.5: Pattern recognition results with k-nearest neighbour (k=5) and k-fold cross validation (k=7). Group Genuine 1 was assigned as representative samples. Integration pattern was based on the approach of weighing the top 4 main constituents, weight: patchoulol = 15, α-bulnesene = 20, α-guaiene = 30, seychellene = 40. Choice was based on high values of Eigenvalues and their explanatory power when no weights were assigned. Group Genuine 1, adulterants and deliberately adulterated are trainings sets. Commercial samples are test sets.

The test set Genuine 2 was correctly assigned to the Genuine 1 training set with the exception of sample 80. Visible inspection of the spectrum did not show any remarkable differences with other samples. Higher number of Genuine 1 as a test set could solve this problem when aiming for 100% security. Also a switch to Genuine 2 as a Training set gave correct assignment of Genuine 1 (not shown). Same accounts for the commercial set were one genuine sample was wrong advised as deliberately adulterated. Again the model had difficulties to assign the two Clearwood samples as their spectra show no remarkable differences. Approaches with weighing patchoulol and pogostone did not yield any satisfactory results. Here commercial genuine samples were not properly assigned. Same holds for the approach of weighing all adulterants to 100%. Also here two commercial genuine samples were not properly assigned (not shown). However, a small percentage of wrong assigned samples can be investigated by visual inspection which still safes time compared to an inspection of all samples.

Besides the sample assignment the focus was on model quality. As the weighing approach with the top 4 contributors showed best results and also highest model quality this was the favoured direction (Fig 3.5 and Tab. 3.2).

Table 3.2: Model quality cross validation results from ChemPattern for pattern recognition of GC data. Model was created with group Genuine 1, Pure adulterants and deliberately adulerated samples. Results are based on the weighing of the top 4 constituents (weight: patchoulol = 15, α-bulnesene = 20, α-guaiene = 30, seychellene = 40). Abbreviations: FRR = false reject rate is a model type I mistake which describes the likelihood that the created model incorrectly rejects a sample which is supposed to be genuine and or adulterated. FAR = false acceptance rate is model type II mistake and describes the likelihood that the created model accepts a sample in a certain group even though its wrong e.g. a genuine sample as deliberately adulterated. The lower FRR and FAR the better the model. NOTE: as model cross validation is enabled the results may vary as with every new recall of the results the cross validation creates new groups (see also 2.7.6).

Group name	Attribute	Total	FRR	FAR
Genuine 1	Train.	31	-	6%
Genuine 2	Test	30	-	-
Commercial genuine	Test	8	-	-
Commercial adulterated	Test	5	-	-
Pure adulterants	Train.	19	5%	-
Deliberately adulterated	Train.	18	6%	-

k numbers	k-recognition accuracy [%]
1	94
2	94
3	97
4	97

The model accuracy was calculated with 97% by ChemPattern. A higher k-number did not result in any notable differences while k < 4 showed a lower accuracy. Mistakes of first and second order (FRR and FAR) were also low. As pattern recognition relies on their data base every additional sample would add value to this library and increase predictability and reduce wrong assigned samples. However, it is shown that its clearly possible to assign a majority of samples to their right group. Again is Clearwood a special case and its proper assignment is impossible for the algorithm as no clear distinction based on the TIC is possible.

This section presented that the “golden standard” GC-MS, is as expected, capable of detecting all kind of adulterants if combined with a semi-quantitative approach. Even though Clearwood was not to differentiate with chemometric screening tools so far, a simple visual inspection of the TIC was enough to identify the additional product of possibly ethylated patchoulol. Visual inspection of a chromatogram the percentage of non-volatiles are probably still preferred nowadays rather than automated evaluation of the data. To evaluate low-field 60 MHz NMR as a complementary technique the same approaches were tried.

3.5 60 MHz ^{1}H NMR analysis

For the 60 MHz NMR analysis the same samples were used as for GC-MS analysis. Figure 3.6 shows common pattern of all relevant genuine PEOs as well as a 600 MHz spectrum of PEO from Robertet. Overall variation within the spectra was small. It is obvious that the 600 MHz spectra displays far more information in great detail (see also Fig 6.4 for patchoulol). Even though preparation time was almost identical with 60 MHz samples as only dilution with $CDCl_3$ was necessary, maintenance, costs and knowledge to operate a 600 MHz system makes it unsuitable for routine QC.

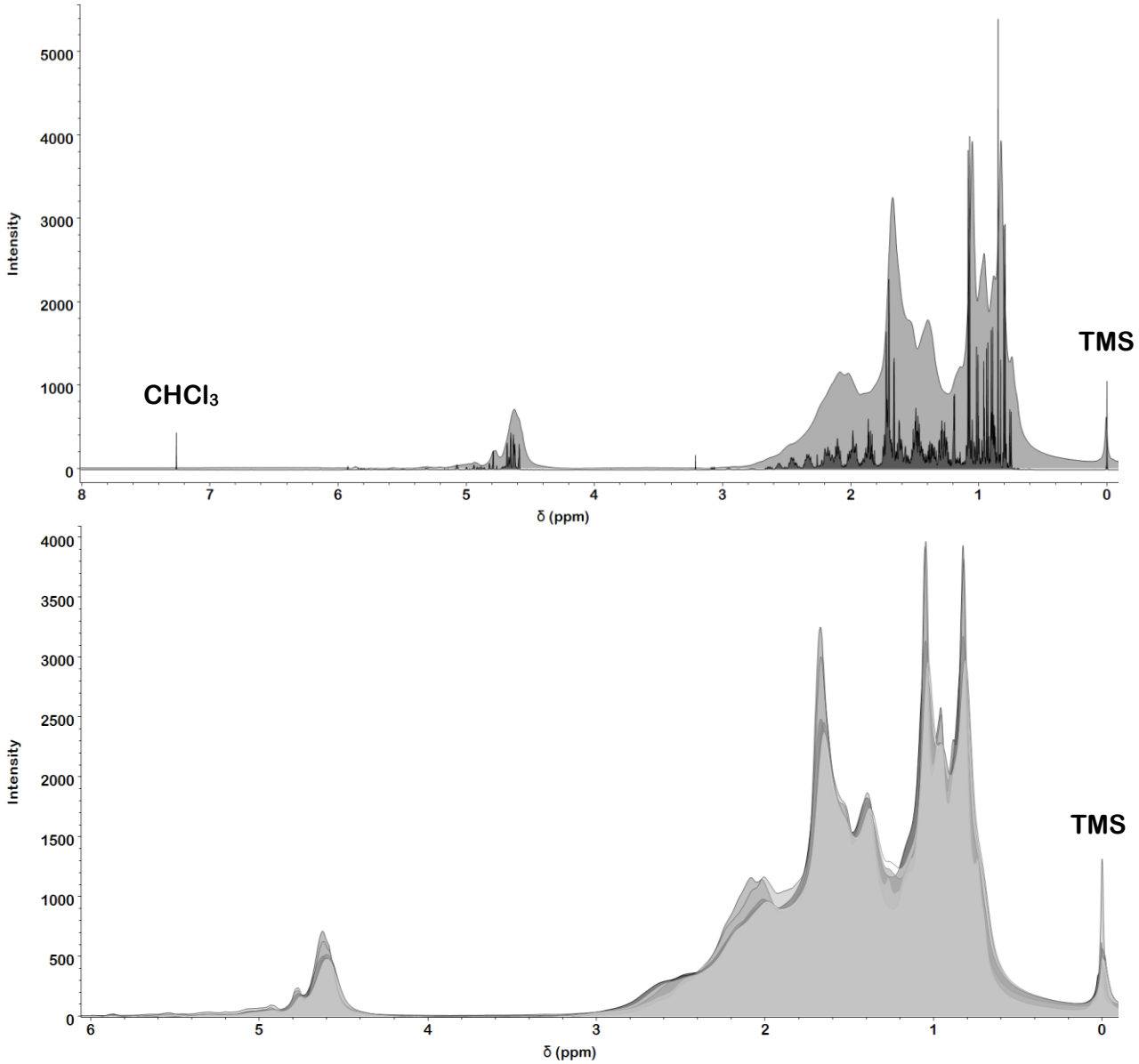


Figure 3.6: Above picture of ^{1}H 60 MHz spectrum of PEO-Robertet vs ^{1}H 600 MHz spectrum of PEO-Robertet. To mention is the slight shift in the 600 MHz spectrum compared to 60 MHz due to $CDCl_3$. Below depicted are 5 different oils to show the natural variability within the PEOs (sample 37, 56, Robertet, 16, 55)

To mention is that a semi-quantification as done with the GC-MS data was not preferred. Sample preparation should remain as easy as possible. Additional weighing of an internal standard would cost additional time and is consequently not desired.

A quick visual inspection of the NMR spectra could already give a good impression if an oil was adulterated or not just like with GC-MS. Additional aromatic compounds (region around 7-8 ppm) could easily distinguish a sample from a genuine oil (see Fig. 3.9). For the majority notable areas appeared while gurjun, paraffin, copaiva and Clearwood were difficult to identify with the human eye (Fig. 3.7).

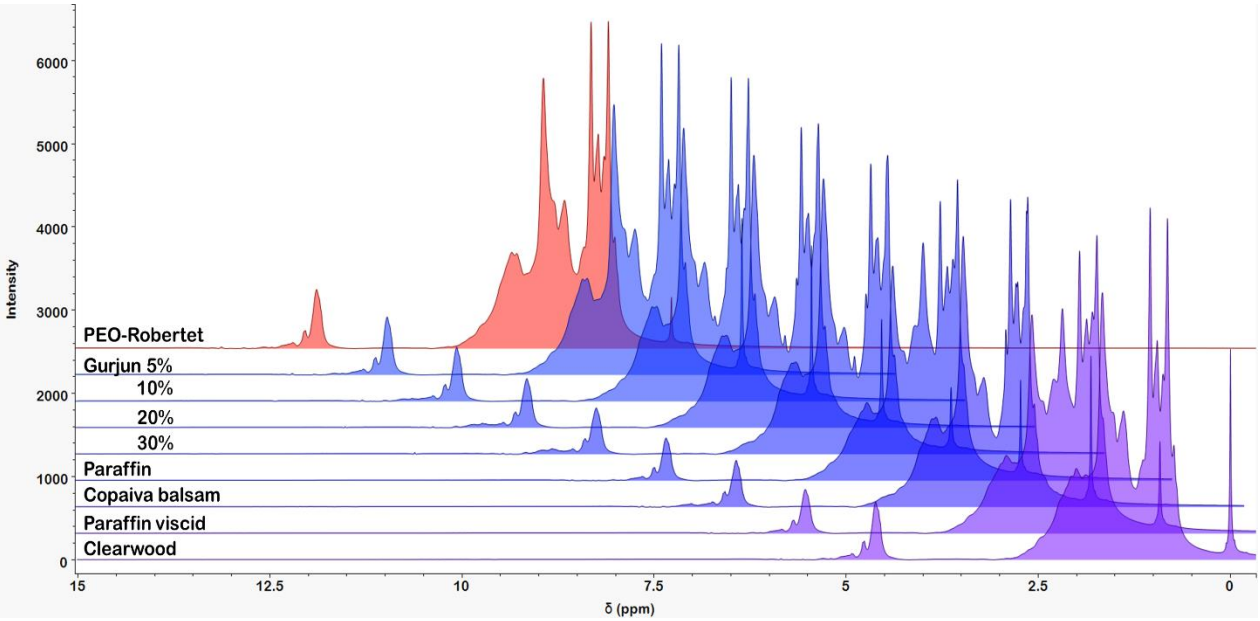


Figure 3.7: Depicted are several adulterated oils on a ^{1}H 60 MHz machine to show the difficulties for interpretation solely based on visual inspection. Spectra are shifted to clarify problem.

3.6 Similarity analysis for NMR

Using the complete band from 0 – 8.1 δ

To get the best possible results several approaches were tested. The first approach was to use the default settings by integrating the whole spectrum from 0 - 8.1 ppm with a band width of 0.01 ppm. The Mahalanobis distance results are shown in Figure 3.8. An important note is that only 4 out of 13 commercial samples are shown due to peak broadening (see section 3.9). Figure 3.8 shows that Group Genuine 2 gives high similarity to group 1. Same accounts for the commercial genuine samples. The sample from Anthemis with Malaysian PEO has clear distinguishing features. Interesting to see is that the gradual adulteration with gurjun balsam from 5 to 30% is nicely depicted even though differences between a genuine oil spectrum and adulterated one were minor and with human eye difficult to detect (Fig. 3.7 - 8).

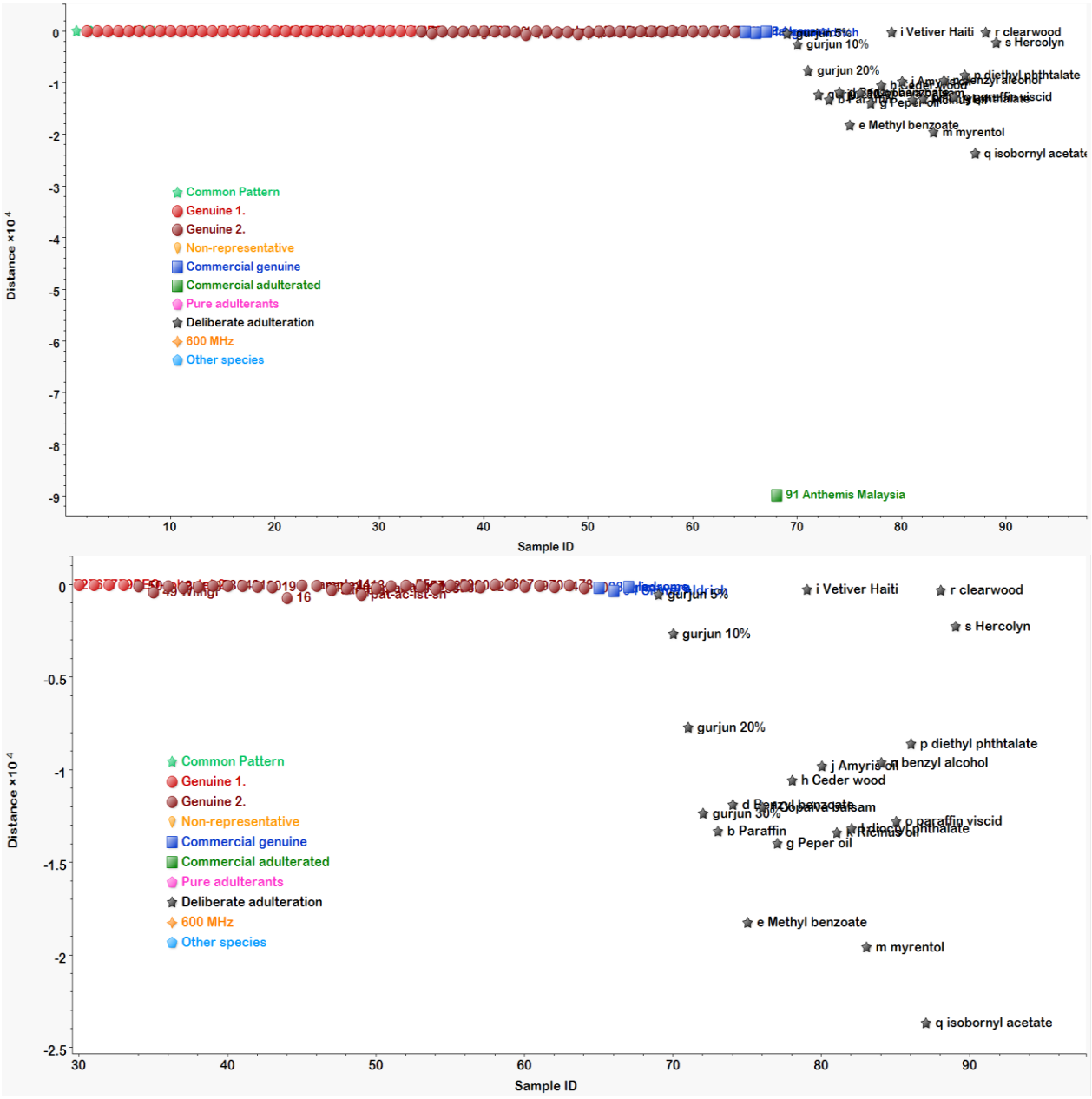
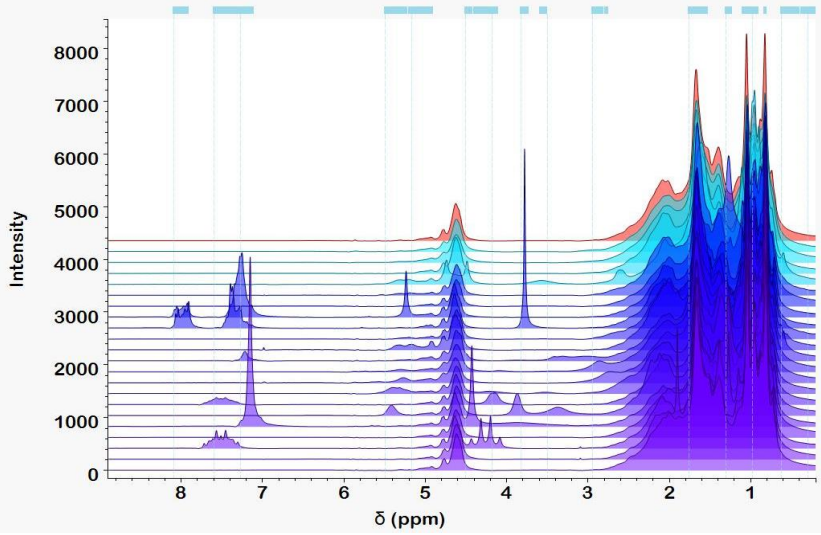


Figure 3.8: Upper: Mahalanobis distance measure results. Integration band region from 0 – 8.1 ppm with integgration band width of 0.01 ppm. Group Genuine 1 was assigned as “representative sample”. Rest was assigned as “normal sample”. Shown are only Genuine 1 and 2, Commercial samples and deliberate adulterated samples. Lower: zoom in into results for the deliberate adulterated samples.

A 5% gurjun adulteration remains on the edge of being distinguishable from genuine samples but will it be hardly conducted in practise as a 5% adulteration does not seem economically useful for PEO. Vetiver and Clearwood remain undistinguished from genuine samples just like in similarity analysis for GC-MS. A problem for vetiver is that the algorithm is probably not taking an additional area at 3 ppm into account (or its weight is not important compared to other regions) while it is easy to detect by the human eye (see Appendix Fig. 6.5). For an additional results with the non-representative group and essential oils from other species see Fig 6.6.

Selective approach with focus on adulterants and crucial PEO regions

A second approach included a more selective analysis to focus on important regions which are crucial for high quality oil or certain adulterants. Regions were chosen based on a 600 MHz sample of pure patchoulol (Fig. 6.3) as well as PEO from Robertet to identify common peaks (Fig. 3.6 above). Additional regions were identified with the loading plots of a multivariant analysis with ChemPattern based on the whole spectrum from 0 to 8.1 ppm with a spectral width of 0.01 ppm. The combined approach yielded following regions (Fig. 3.9). As shown in Figure 3.10 the results are quite similar to the previous whole spectra approach but distinction could be refined which is especially visible with benzyl and methyl benzoate but also accounts for the other adulterants. Still natural variation is a challenging factor which may lead to wrong assignment of samples to genuine or adulterated as it can be seen with samples 16 (highlighted with arrow). Results may be better for this approach but it is only based on known adulterants and samples. As already mentioned the same problem is inherit to GC-MS. Distinction is improved but new samples/adulterants could remain undetected. This approach is more likely when a bigger database of several oils is available.



	Chemical shift [δ]
Low field	8.1 - 7.9
	7.6 - 7.1
	4.9 - 5.5
	4.5 - 4.1
	3.825 - 3.725
	3.6 - 3.5
	2.95 - 2.75
	1.77 - 1.52
	1.31 - 1.22
	1.1 - 0.9
	0.84 - 0.8
High field	0.63 - 0
Spectral width	0.01

Figure 3.9: Integration bands for the ^{1}H 60 MHz NMR spectra in light blue above the spectrum with 0.01 ppm band width. Important regions have been identified with loading plots and by visual inspection of spectra. Regions which were not depicted in the loading plot have been added manually in terms of importance. Group Genuine 1 served as "representative samples". On the right side the exact chemical shifts. Depicted in the picture are PEO-Robertet in orange. Commercial samples in light blue/green. Deliberately adulterated samples in deep blue to purple.

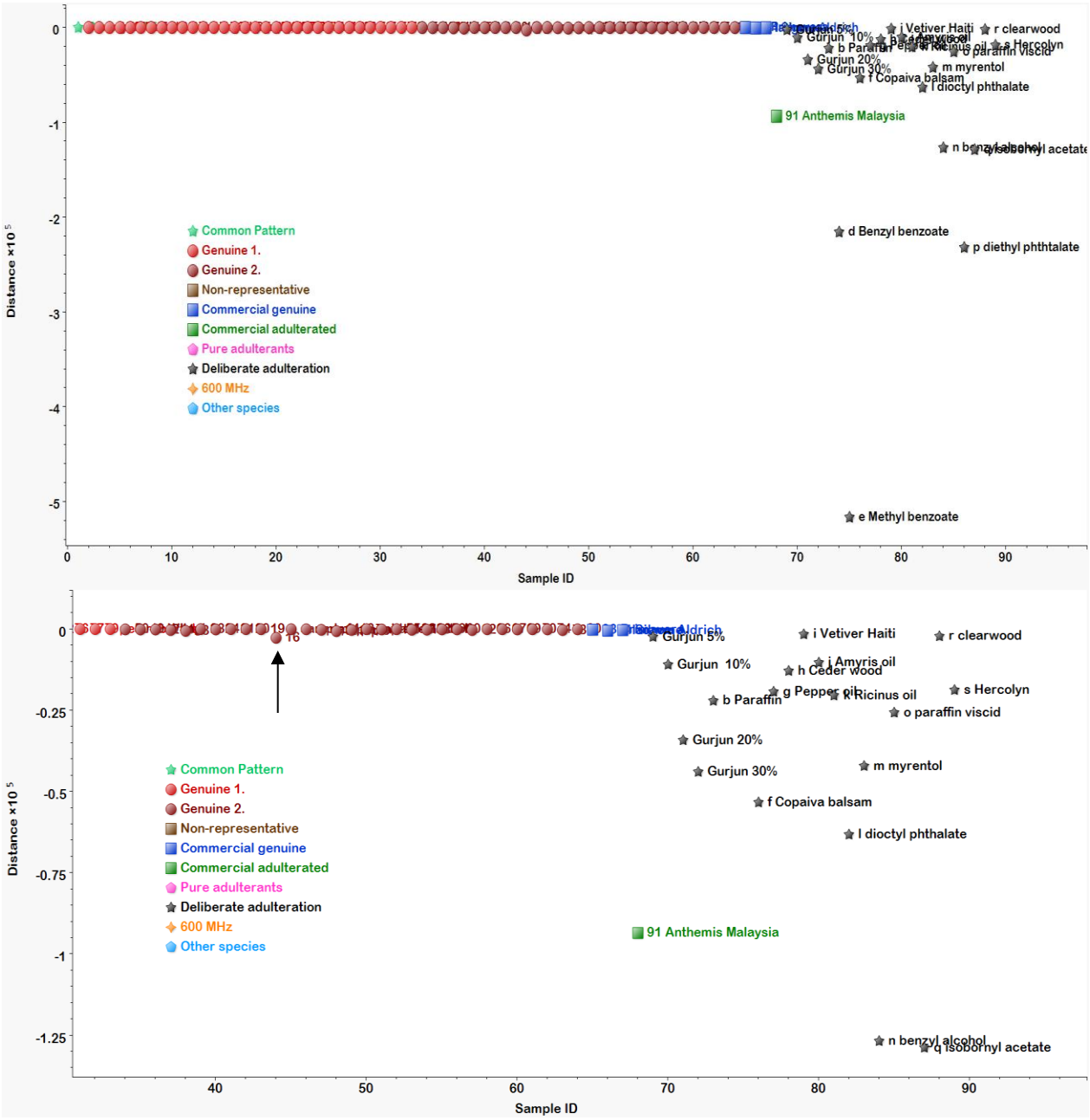
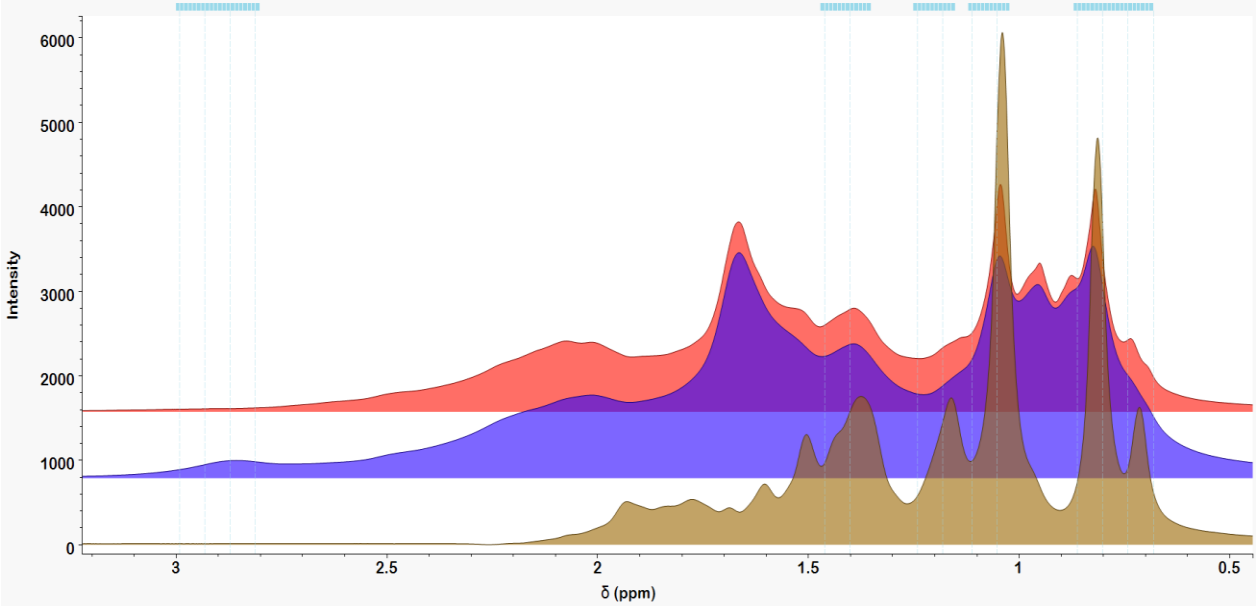


Figure 3.10: Upper: Mahalanobis distance measure results. Integration band region based on important PEO and adulterant regions as shown in fig. 3.7 with peak width of 0.01. Group Genuine 1 was assigned as "representative sample". Rest was assigned as "normal sample". Shown are only Genuine 1 and 2, Commercial samples and deliberate adulterated samples. Lower: zoom in into results for the deliberate adulterated samples.

Targeting inherent PEO traits for best results - patchoulol

The last approach was to focus on the main constituent patchoulol. Substance was available in pure form and was analysed on a 600 MHz and 60 MHz machine (Fig 6.4). Peaks were identified, compared and yielded following integration regions (Fig. 3.11). Figure 3.12 shows that distinction is now clearly possible by integrating patchoulol and vetiver regions. While the integration bands for patchoulol distinguish the majority of adulterated samples is it not enough to differentiate for vetiver. This led to inclusion of the region from 2.8 – 3 ppm. Some natural variation in the group Genuine 2 is still within acceptable limits. 5% gurjung and Clearwood adulteration remain indistinguishable. The additional by-product during the Clearwood production which was visible on 600 MHz cannot be resolved on a 60 MHz machine. Previous attempts to distinguish Clearwood adulteration based on pogostone failed as the content for pogostone was not suitable as an indicator. Natural variation of pogostone content varies to a considerable amount. A 600 MHz spectrum of pogostone was recorded and assigned (Fig. 6. 7).



	Chemical shift [δ]	Causing substance
Low-field	2.8 - 3	vetiver oil
	1.35 - 1.47	patchoulol
	1.15 - 1.24	patchoulol
	1.02 - 1.12	patchoulol
High-field	0.68 - 0.87	patchoulol
Spectral width	0.01	

Figure 3.11: Integration bands for the ^{1}H 60 MHz NMR spectra in light blue above the spectrum with a band width of 0.01 ppm. Important regions have been identified with analysis of pure patchoulol and an adulterated sample with vetiver on 600 MHz and/or 60 MHz. Region from 2.8 – 3 ppm is caused by vetiver. Lower table shows the exact chemical shifts. 1) in red is PEO-Robertet. 2) in blue deliberately adulterated sample with vetiver. 3) pure patchoulol in brown.

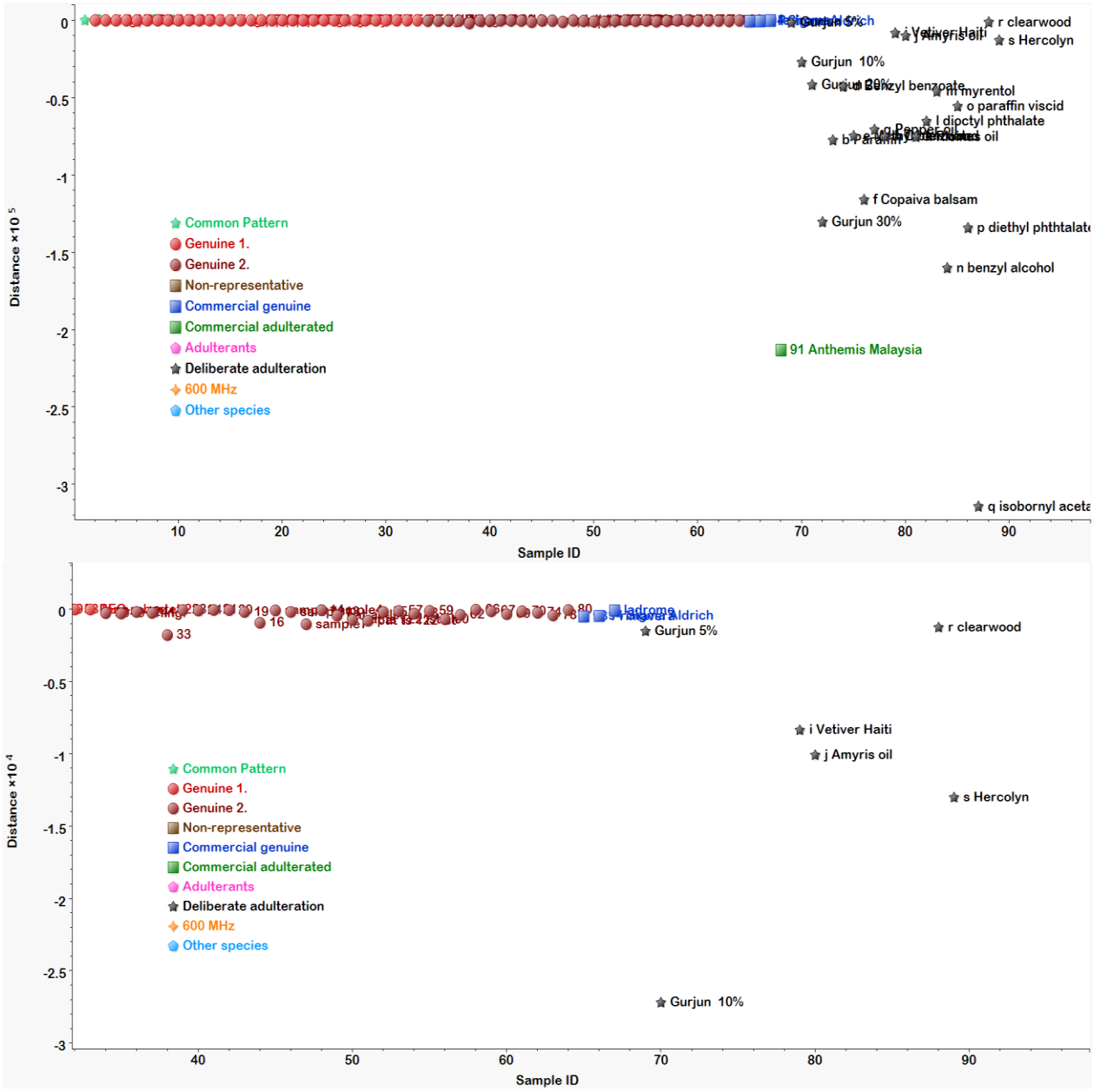


Figure 3.12: Upper: Mahalanobis distance measure results. Integration band region based on patchoulol and vetiver adulteration. Integration regions as shown in fig. 3.10 with peak width of 0.01. Group Genuine 1 was assigned as "representative sample". Rest was assigned as "normal sample". Shown are only Genuine 1 and 2, Commercial samples and deliberate adulterated samples. Lower: zoom in into results for the deliberate adulterated samples.

The similarity analysis results for the NMR spectra showed that it is again not possible to distinguish Clearwood adulteration from genuine samples. The advantage of the last approach lies in the distinction which is relying on PEO properties itself. It rather detects low and high quality oils than adulteration itself. Similar results were obtained with PCA for GC-MS (Fig. 3.4). Nonetheless, vetiver could not be distinguished without the additional region from 2.8 – 3 ppm (see also Fig. 6.5). New adulterants may have similar features like vetiver and cannot be separated only on the basis of patchoulol. Specific integration may miss new adulterants (Figure 3.9 – 10) but the whole region integration from 0 – 8.1 ppm has its power that no discrimination takes place. The current software package of ChemPattern is not providing a weighing factor but with the combined knowledge from the other approaches, the results could maybe even improved for clearer distinction. The focus here should also lie on patchoulol and further regions only as additive aid. For now approach three with focus on patchoulol and vetiver would be most feasible in practise. With sufficient big library a switch to approach two (adulterant specific) is advised for distinct results.

3.7 Multivariate analysis for NMR

Multivariate analysis failed to give any useful results. No distinction was achieved with any of the previous integration methods. Complexity of the spectrum may be an explanation as with a band width of 0.01 ppm a tremendous amount of variables has to be taken into account. Results could not be optimised with a weighing factor as done with GC because it is not implemented yet in ChemPattern version 2.0.

3.8 Pattern recognition for NMR

For the pattern recognition of NMR spectra the test set of commercial samples was reduced as non-sharp and broad spectra prevented data analysis. Results are shown in Figure 3.13 for whole region integration. One sample from the genuine 1 group could not be assigned. The whole genuine 2 test set was correctly assigned. Also changing the training set to genuine 2 still gave the same results with 1 wrong assigned sample. Even though the commercial test set was reduced to only four samples, they were assigned correctly including Anthemis Malaysia as heavily adulterated PEO. Similar results were obtained with the other two approaches (important regions of PEO and adulterants, patchoulol and vetiver). If deliberately adulterated samples were set as test set they were for all approaches assigned as genuine. Single adulteration and the high quality PEO as basis are the reasons that the distinctive features are not enough for the created model. However, adjustment of k-parameter was necessary to improve further results. It is observable that with a more simple integration approach k-number has to increase to maintain proper distinction features (Table 3.3).

Although results were all similar, statistical results showed that the prediction accuracy and proper sample assignment differed within the approaches (Table 3.3). The algorithm specifically struggled in assigning adulterants and deliberately adulterated samples which may be caused by the low number of samples (visible by a slight shift). Every adulterant is a single unique sample compared to the bulk of genuine samples which made it easy to assign these. Accuracy of the models were similar between the different approaches (86%, 84%, 86% respectively). Why the two genuine samples are not properly assigned is unknown. Lower model accuracy than the GC model with 97% could be a reason. It is noteworthy that these numbers may vary as the k-fold cross validation arranges every time new folds which influences the results. Differences are minor between results. As the pattern recognition function is a decision making tool for quality control, the false reject or false acceptance rates are still considerably high for that purpose compared to the GC results (Table 3.2). But it should not be neglected that the sample size is small. As mentioned beforehand for the GC result is the pattern recognition just as powerful as the underlying dataset. With disregard to the sample size, NMR has potential in detecting high or low quality PEO's just as good as GC-MS.

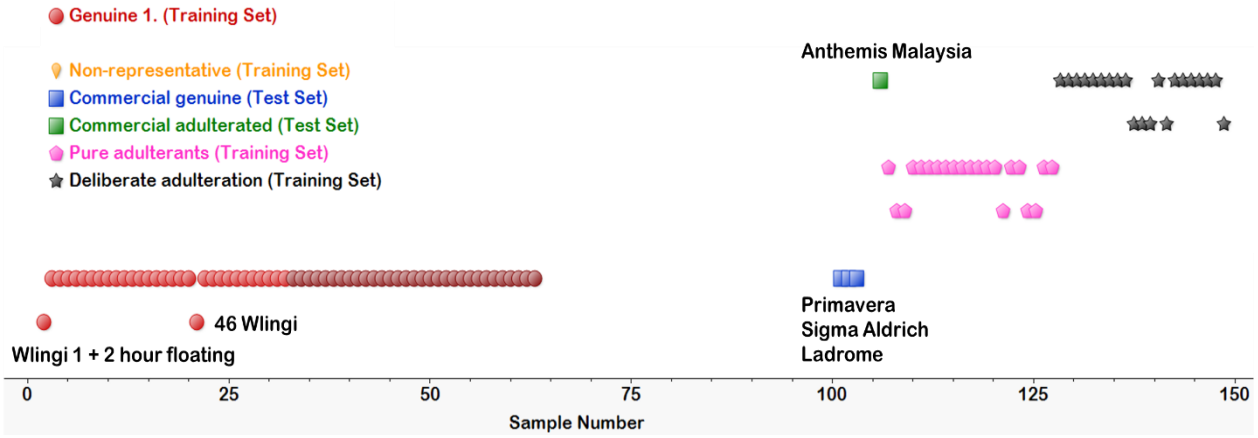


Figure 3.13: Pattern recognition results for NMR with k-nearest neighbour (k=3) and k-fold cross validation (k=7). Model validation showed accuracy of 86%. Common pattern based on whole integration from 0 – 8.1 δ. Group Genuine 1 was assigned as representative samples. Group Genuine 1, adulterants and deliberately adulterated are trainings sets. Commercial samples are test sets.

Table 3.3: Statistical results of pattern recognition. Shown are the analysed groups and their assigned function (training or test set). Abbreviations: Whole region = Integration from 0 – 8.1 δ. Important region = integration parameter referring to 3.9. pat. = patchoulol, vet = vetiver oil integration refers to 3.11. Results in upper table show how many samples were accurate assigned with the number of false positives or false negatives. Lower table shows how accurate the created model by Genuine 1, Pure adulterants and Deliberately adulterated samples was. **False reject rate** is a model type I mistake which describes the likelihood that the created model incorrectly rejects a sample which is supposed to be genuine and or adulterated. **False acceptance rate** is model type II mistake and describes the likelihood that the created model accepts a sample in a certain group even though its wrong e.g. a genuine sample as deliberately adulterated. The lower FRR and FAR the better the model.

			False reject rate			False acceptance rate		
Group name	**Attribute**	**Total**	**Whole region**	**Important region**	**pat. + vet.**	**Whole region**	**Important region**	**pat. + vet.**
Genuine 1	Train.	31	6%	3%	-	19%	38%	-
Genuine 2	Test	31	-	-	3%	-	-	13%
Commercial	Test	4	-	-	-	-	-	-
Pure adulterants	Train.	21	24%	33%	19%	-	5%	5%
Deliberately adulterated	Train.	21	24%	48%	38%	29%	24%	29%

k-recognition accuracy [%]			
k	**Whole region**	**Important region**	**pat. and vet.**
1	86	84	86
2	86	84	86
3	82	74	85
4	-	77	85
5	-	77	86

3.9 Metal extraction against peak broadening

Some spectra for NMR analysis (PEO as well as other oils) showed peak broadening and were unsuitable for analysis. We hypothesized that metal contamination caused by the distillation process with non-stainless steel could cause this phenomenon. Most of the oils with a broad spectrum had a darker colour compared to the high quality genuine oil from Indonesia or Madagascar which was yellow/clear while the broad samples were orange to dark orange. Contradictory to that was that some broad spectrum samples were yellow/clear. To investigate this, metal extraction was conducted with EDTA as described in §2.5. A commercial sample from De Tuinen was chosen due to its broad spectrum. From GC-MS analysis it was already known that this sample is genuine. Figure 3.14 shows that metal contamination is not the cause of band broadening in NMR analysis.

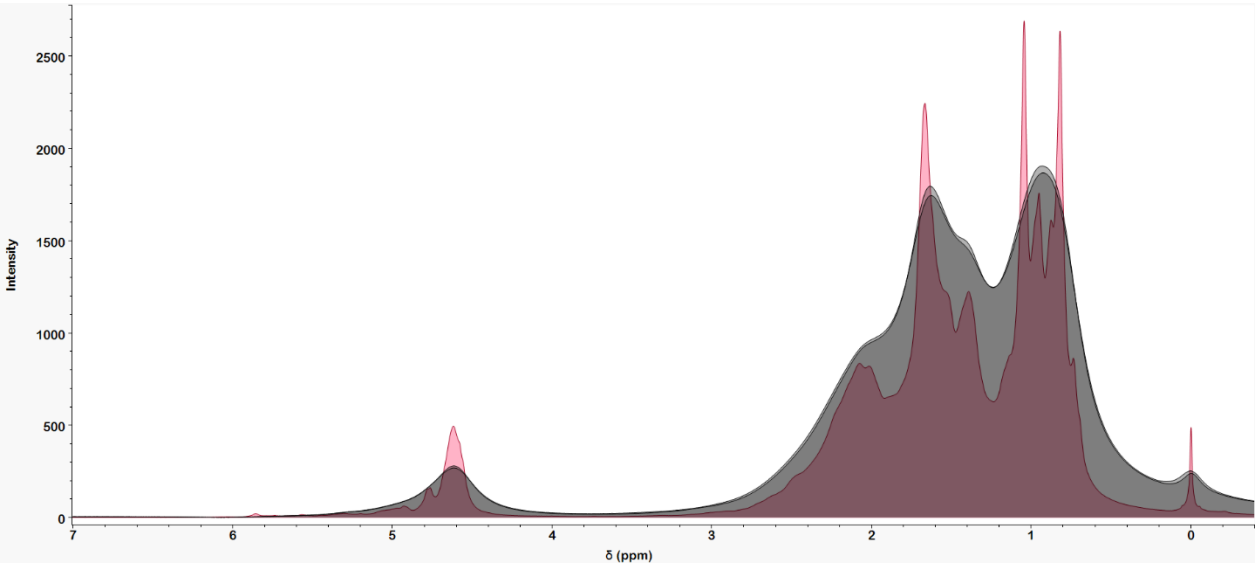


Figure 3.14: ^{1}H 60 MHz overlap profile of De Tuinen before metal extraction (in the background in light grey) compared to De Tuinen after metal extraction (in dark grey). Red sample is PEO from Robertet to indicate a proper spectrum quality with no broadening.

3.10 Dilution against peak broadening

After metal showed not to be the problem of peak broadening, a simple dilution with 10% oil in 90% $CDCl_3$ was conducted. Again De Tuinen served as genuine commercial oil as test sample. Indeed could a simple dilution solve the problem of peak broadening as seen in Fig. 3.15. Besides a lower intensity/signal amplitude the samples does also have a clearer and sharper spectrum as the undiluted approach. It was not conducted to what extent dilution is necessary to eradicate peak broadening. Further experiments could investigate this. Maybe a 50% dilution or even less is necessary. Viscosity itself can be excluded as most of the commercial samples who showed peak broadening had low viscosity. However, while previous results showed that non-diluted samples for NMR analysis seem to have enough resolution to separate genuine from adulterated samples could an overall approach of dilution for NMR further improve distinction results as spectra are sharper. These newly created spectra cannot be used for the current library of oils. The undiluted common pattern does now allow comparison with the new samples which are lower in intensity and different overall spectrum. Disadvantage are then the increased time costs which makes NMR preferable as weighing like in GC-MS has to be conducted.

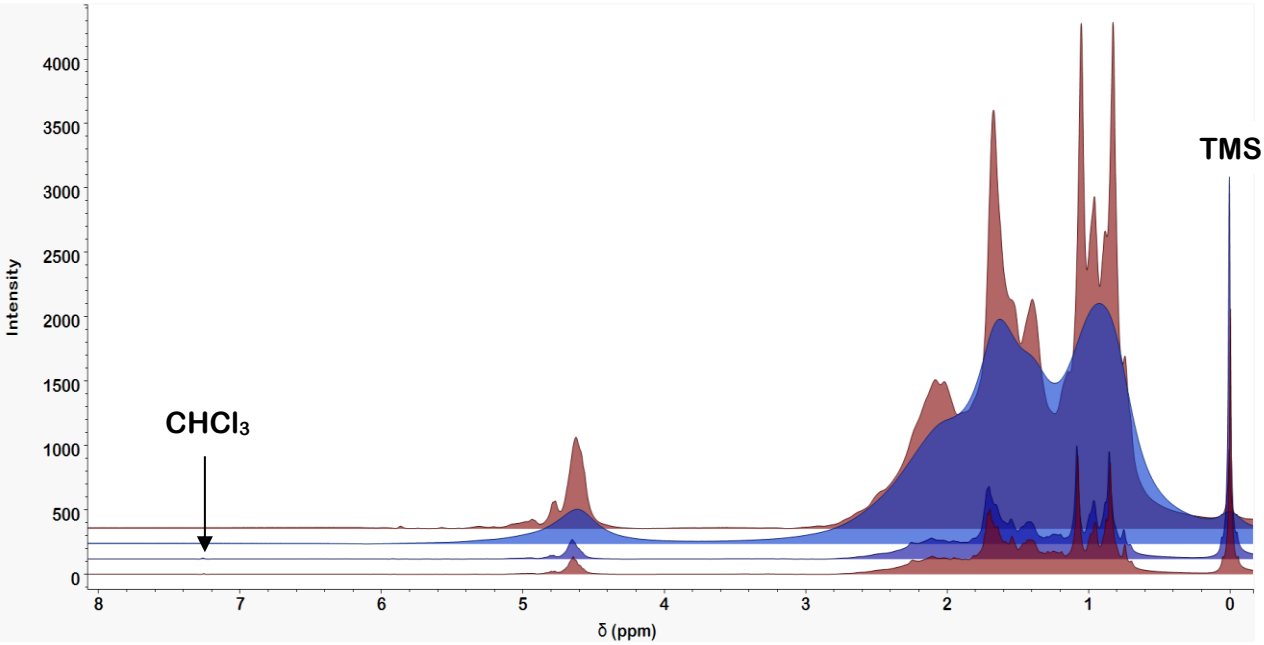


Figure 3.15: ^1H 60 MHz NMR of (blue) the commercial genuine oil De Tuinen. Broad spectrum shows sample before dilution, small sharp spectrum after dilution with 90% $CDCl_3$. Red genuine PEO from Robertet before and after dilution.. Indicated are the TMS peak at 0 as chemical shift reference and $CHCl_3$ residues from $CDCl_3$.

3.11 Refractometry

Besides today's sophisticated QC techniques the old method of refractometry was applied to test whether more simple and easy alternatives also yield sufficient results. According to a summary of four different reports genuine PEO is characterised by a refractive index of 1.503 – 1.513 (van Beek and Joulain unpublished). Results from Table 3.4 indicate that all measured genuine samples from Indonesia, PEO from Robertet and commercially genuine samples are within that range. Yet half of the deliberately adulterated samples are also within that range which makes refractometry unsuitable for a first QC tool. Nevertheless this technique demonstrates that for certain adulterants refractrometry is an appropriate tool. Especially when looking at non-volatiles like paraffin where GC-MS has its difficulties to detect it is refractometry the easy and cheap alternative.

Table 3.4: Refractive indices for indicated samples. In red marked are indices which are out of the range from 1.503 – 1.513. Green marked are values within the range. Deliberately adulterated samples are adulterated with 20% of indicated adulterant in background of PEO from Robertet if not stated differently.

Representative samples	Refractive index
PEO-Robertet	1.5070
17	1.5099
4	1.5070
78	1.5076
non-representative	
64	1.5070
9	1.5061
6	1.5120
Commercial genuine	
De Tuinen	1.5072
Jacob Hooy (new)	1.5072
ladrome	1.5072
Commercial adulterated	
Anthemis Malaysia	1.5015
Carl Roth	1.5091
F.E.S Rotterdam	1.5205

Deliberately adulterated	Refractive index
amyris oil	1.5042
benzyl alcohol	1.5090
benzyl benzoate	1.5152
cedar wood oil	1.5042
Clearwood	1.5009
copaiva balsam	1.5050
diethyl phthalate	1.5035
dioctyl phthalate	1.5000
gurjun balsam 5%	1.5058
10%	1.5035
20%	1.5025
30%	1.5020
Hercolyn	1.5061
isobornyl acetate	1.4956
methyl benzoate	1.5056
paraffin	1.4982
paraffin viscid	1.4975
pepper oil	1.5000
propyleneglycol	1.5009
ricinus oil	1.4980
vetiver oil	1.5084
1R - (-) myrtenol	1.5025

4 Conclusions and recommendations

In this study the potential for a 60 MHz NMR bench-top spectrometer was evaluated compared to the already validated and known method for essential oil QC, GC-MS. The overall results are summarized in Table 4.1. Even though GC-MS has high costs for the machine itself including gas, the reduced accessibility as skilled technicians are required to run and operate the system, the accuracy the main argument for GC-MS as golden standard QC-tool. This fact allowed to create a reliable library of the analysed oils.

Table 4.1: Summarized results of the research. Red marks unsuitable for QC issues. Yellow indicates intermediate usefulness and considerations should be taken into account. Green marks high suitability for QC. Highlighted in bold is accuracy as the most crucial aspect in quality control. Costs refer to the costs for the machine itself as well as running costs and sample preparation costs. Space refers to the space needed to install the equipment and further necessities like a computer. Accuracy to the sensitivity to detect adulterants with chemometrics. Accessibility to operate and maintain the system. Time refers to the overall time needed for sample preparation and sample analysis/run.

Attribute	GC-MS	low-field NMR	Refractometry
Costs			
Space			
Accuracy			
Accessibility			
Time			

As expected GC-MS was capable of detecting all kind of adulterations including non-volatile compounds which could be revealed by semi-quantification (Table 3.1). The identified by-product from Clearwood at 21.44 min with a mass of m/z = 250 is with applied approaches not significant enough to account for distinction (Fig. 6.2). However, visible inspection of the spectrum can show quickly if an adulteration with Clearwood was performed or not. At the time when this study was conducted Clearwood adulteration was not yet mentioned in literature.

For the NMR spectra the similarity analysis based on Mahalanobis distance measure showed good results with clear distinction just like GC-MS. Best results were obtained with an integration approach including patchoulol and vetiver regions (Fig 3.11). This shows that the best distinction was achieved by focusing on small crucial regions from PEO itself rather than include too much information. Clearwood and 5% adulteration with Clearwood remained undetectable. Pattern recognition results for GC (Fig. 3.5) showed high accuracy (97%) and low rejection rates of samples (Tab. 3.2) which is crucial for practical QC purposes. Instead NMR results were less assuring compared to GC-MS pattern recognition. Prediction accuracy was in general 10% lower (around 86%) and mistakes of first and second order were considerably high (Table 3.3).

Despite excellent adulteration detection both methods GC-MS and NMR were capable of distinguishing between high and low quality oils (Fig. 3.2, 3.12) which was further optimised with PCA for GC-MS (Fig 3.3).

The encountered problem of peak broadening, which was first assigned to metal contamination during the distillation process (Fig 3.14), could be solved by dilution of those samples (Fig 3.15). The advantage of quick sample preparation is abolished if dilution is necessary for a significant amount of samples.
Another important aspect, which was not mentioned yet, is the environment in which the NMR is placed. Switching temperatures exacerbates constant results which resulted in changing intensities of the spectra and subsequently influenced statistical analysis.
With disregard to that 60 MHz low-field NMR is an excellent QC-tool. All known adulterants were detected for reasonable costs. No costly gas installations nor much space is needed for the bench-top 60 MHz NMR. Operation of the system was easy and straightforward which also resulted in short preparation and measuring times.
At this point in time, low-field NMR potential for QC in the essential oil sector looks promising. However, it remains to clarify what the source of peak broadening is and to what extent the time consuming step of dilution needs to be done.
Marks should also be taken for the refractometry. Although accuracy was by far not enough with about half of the adulterants could not be detected, convinced the simplicity of the tool and its ability to give results in less than one minute. Non-volatiles (paraffin) which were more difficult to detect by GC as they are not depicted in the TIC can be easily proven by refractometry which makes it a good and very cheap complementary tool for essential oil QC (Table 3.4).
As this research made excessive use of Chemometrics with ChemPattern, some notes should also be taken here. A weighing factor was not implemented yet for NMR analysis but according to the developer it will be integrated in the next software update. This could facilitate the creation of a useful PCA to be able to identify high and low quality oils just like GC-MS when combined with a quantitative approach. Same holds for a decisive tool for distinction, namely additional peaks/areas, which are not in the common pattern to immediately mark these samples as outlier/adulterated. This feature would tremendously ease separation and distinction of samples as most of the adulterants showed additional peaks/areas.

Acknowledgement

I would like to thank my supervisor Teris for taking again a last master student. Excellent and relaxed supervision while giving hints and incentives on how to work and research. Alex Tian from Chemmind Technologies for helping understand the software. Frank, Barend and Elbert for being open to various question for all kinds of every day lab things and Pieter de Waard for helping me to measure on a 600 MHz machine.

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6 Appendices

Table 6.1: Analysed patchouli oils obtained from Indonesia. # = number of sample. n. rep. = not representative and subsequently not used for analysis. Date = production date of oil in Indonesia. Some samples are without a sample number (#) as they were analysed later.

#	n. rep.	Oil	Name / Properties / code	Date	Density	Origin	Colour
1	x	Patchouli	GHR 3 Hydrodist after 12 hours infusion 1ml/min	3/2/2000		Padang	Dark orange
2	x	Patchouli	Hydrodist 12hours infusion				Orange
3	x	Patchouli	6 hours Hydrodist, 12 hours infusion, 1ml/min SO4	3/7/2000			Yellow clear
4		Patchouli	Chandra	2000			Yellow
5		Patchouli	6 hours Hydrolist 1ml/min CaCO3	3/4/2000			Yellow
6	x	Patchouli	VIII 05 Senali	3/2/2000		Bengkulu Distillation	Orange
7		Patchouli	Patchouli Nov - Dec	x.x.2000			Yellow
8	x	Patchouli	VII DS. Kurotidur unit III	3/2/2000		Bengkulu Distillation	Yellow
9	x	Patchouli	WL 5/1	7/31/2006	0.948		Yellow
10		Patchouli	WL 5/2	7/31/2006	0.95		Yellow
	x	Patchouli	WL 5/3	7/31/2006	0.952		Yellow
11		Patchouli	WL 5/4	7/31/2006	0.96		Yellow
12		Patchouli	WL 5/5	7/31/2006	0.966		Yellow
13		Patchouli	WL 5/6	7/31/2006	0.966		Yellow
14		Patchouli	WL 5/7	7/31/2006	0.968		Orange
15		Patchouli	Trenggalek Brown	10/12/2006			Yellow clear
16		Patchouli	Wilingi, 1st + 2nd hour, emulsified oil	2/27/2007			Yellow
17		Patchouli	Tempursar ACEH-RS-SD	5/25/2007			Yellow clear
18		Patchouli	AC/LST-SH, IST- Suradaya	x.08-2000			Yellow clear
		Patchouli	Wilingi 1st + 2nd hour Floating oil	2/27/2007			Yellow
19		Patchouli	Straight oil, 6 hours Hydrodist. / Na2SO4	x.03.2000		Padang	Yellow clear
20		Patchouli	Straight oil, 6 hours Hydrodist. / Na2SO4, 1ml/min	3/6/2000		Padang	Yellow clear
21		Patchouli	Straight oil, 6 hours Hydrodist. / Na2SO4	x.02.2000			Yellow clear
22	x	Patchouli	Banyuwangi, BY-1B	7/20/2005	0.974		Dark orange
23	x	Patchouli	Tulungagung 2 TA-2C	8/20/2005	-		Dark orange
24		Patchouli	Wlingi leaves, WL 5/	10/15/2006			Yellow
25	x	Patchouli	Nija-1 --> other species? Alduterated?	x.06.2006	0.942	Java	Dark Yellow
26		Patchouli	Situbonfo, Pat-STB-I1	5/23/2007			Yellow clear
27		Patchouli	Situbonfo, Pat-STB-IA	5/23/2007			Yellow clear
28		Patchouli	Tempursari PAT-TS-3	5/19/2007			Yellow clear
29		Patchouli	Tempursari PAT-TS-1	4/5/2006			Yellow clear
30		Patchouli	Tempursari PAT-TS-2A	5/20/2007			Yellow
31		Patchouli	Tempursari PAT-TS-2	5/20/2007			Yellow
32		Patchouli	Tempursari PAT-TS-3A	5/19/2007			Yellow clear
33		Patchouli	Surabaya, Acem brown leave, SB-ACBR	10/14/2006			Yellow clear
34		Patchouli	Surabaya, Acem green leave, SB-ACGR	10/14/2006			Yellow clear
35	x	Patchouli	Lumatang leaves, LJ-1	10/6/2006			Yellow clear
36	x	Patchouli	Wlingi Stalks, WL-5/	10/20/2006			Yellow clear
37		Patchouli	Trenggalek Green leaves, TG-GR	10/12/2006			Yellow clear
38		Patchouli	Wlingi, 290-360min, WL-4/5	3/11/2006	0.965		Yellow
39		Patchouli	Wlingi, WL-3A	3/4/2006	0.982		Dark orange
40		Patchouli	Wlingi Filtrate PP, WL-4/5A	3/11/2006	0.944		Yellow
41		Patchouli	Wlingi,74-144min, WL 4/2	3/11/2006	0.96		Yellow
42		Patchouli	Wlingi WL-4/3	3/11/2006	0.964		Yellow
43	x	Patchouli	Wlingi WL-3B	3/4/2006	0.984		Dark orange
44	x	Patchouli	Wlingi, 11-73min, WL-4/1	3/11/2006	0.938		Yellow
45	x	Patchouli	Wlingi,Dist. First 10min, WL-4/0	3/11/2006	-		Yellow
46		Patchouli	Wlingi, Detergent extra pp, WL-4/5B	3/11/2006	-		Orange
47	x	Patchouli	Wlingi, WL-2B	3/4/2006	0.974		Dark orange
48	x	Patchouli	Wlingi, WL-2A	3/4/2006	0.97		Dark orange
49		Patchouli	Wlingi, WL-2	3/4/2006	0.95		Orange
50		Patchouli	Wlingi, 218-289min, WL-4/4	3/11/2006	0.966		Yellow
51	x	Patchouli	Nganjuk, NG-1B	4/29/2006	-		Dark orange
52	x	Patchouli	Malang, ML-1A	5/25/2005	-		Orange
53		Patchouli	PAT-AC/LST-Fresh, Sixxon-IST-Surabaya	x.09.2008			Yellow clear
54		Patchouli	Code E3	8/16/2009			Yellow
55		Patchouli	Code E1	8/14/2009			Yellow
56		Patchouli	Code E4	8/17/2007			Yellow
57		Patchouli	Code E5	8/18/2009			Yellow

Table 6.1 continued

#	n. rep.	Oil	Name / Properties / code	Date	Density	Origin	Colour
58		Patchouli	TS/SH, ST/L=86/14	3/26/2009	0.958		Yellow
59		Patchouli	PAT-TS / 122LD	x.04.2008 II			Yellow clear
60		Patchouli	TS/SH/PLANT	10.03.2009 II	0.954		Yellow clear
61		Patchouli	Code E2	8/15/2009			Yellow
62		Patchouli	PAT-TS/122 LD79, 7th - 9th hour	x.04.2008			Yellow clear
63		Patchouli	PAT-TS/122 Top 79, 7th09th hour	x.04.2008			Yellow clear
64	x	Patchouli	PAT-AC/ST Simon IST Surabaya	Aug-Sept. 2008			Yellow
	x	Patchouli	PAT-TS/BD3 SH-P.B. 40-60	x.09.2008			Yellow clear
65	x	Patchouli	E/10				Orange
66		Patchouli	WL 5/5				Yellow
67		Patchouli	TS/LST/5-6				Yellow clear
68	x	Patchouli	TA-2B				Black
69		Patchouli	TS/LST/9-11	4/17/2010	0.954		Yellow clear
70		Patchouli	TS/LST/7-8	4/17/2010	0.956		Yellow clear
71		Patchouli	TS/LST/1-2	4/17/2010	0.95		Yellow clear
72		Patchouli	TS/LST/3-4	4/17/2010	0.959		Yellow clear
73	x	Patchouli	Stems, ST.	3/10/2011		Karlsruhe	Yellow clear
74		Patchouli	Leaves + stems, LST	3/21/2011		Karlsruhe	Yellow clear
75	x	Patchouli	Stems, St.	3/5/2011		Karlsruhe	Yellow
76		Patchouli	TS. LST/7-8	4/17/2010	0.956		Yellow clear
77		Patchouli		4/17/2010	0.957		Yellow clear
78		Patchouli	TS/LST/1-2	4/17/2010	0.958		Yellow clear
79		Patchouli	TS/LST/3-4	4/17/2010	0.959		Yellow clear
80		Patchouli	TS/LST/9-11	4/17/2010	0.954		Yellow clear
81	x	Patchouli	Tempursari Code E/8	12/30/2009	0.965		Yellow
82	x	Patchouli	Tempursari Code E/9	12/31/2009	0.964		Yellow
83	x	Patchouli	Tempursari Code E/7	12/29/2009	0.961		Yellow
84	x	Patchouli	Tempursari c/2 in E/10	1/3/2010			Dark Yellow
		Patchouli	PAT-TS/122 LST	x.03.2008			Yellow clear
		Patchouli	PAT-TS/122 LST/SH	x.04.2008			Yellow clear

Data pathway for GC-MS raw data:

GC-MS Computer in lab 7029 left side, right GC.

Computer -> Windows (C) --> msdchem --> 1 --> data --> Andre K

Note: To import data into ChemPattern converting into AIA format is necessary. Done via MS profiler

Data pathway for GC-MS raw data:

Teaching 60 MHz NMR in Orion

Desktop --> to NMR data --> all samples with tag AK_

Table 6.2: Observed peaks for adulterants which are still visible at 20% adulteration in genuine PEO from Robertet. Bold numbers in m/z indicate important fragments. First ion is parent ion. Note: “Anthemis” adulterant only found in Anthemis samples. Some notes for gurjun and copaiva balsam as two of their compounds are marked with aromadendrane. These compounds contain a cyclopropyl group which is an immediate indicator of adulteration as the metabolism of *Pogostemon cablin* is not producing such compounds.

Adulterant	Elution [min]	m/z	Comment
1R - (-) myrtenol	6.15	152, 134, 119, 108, 91, 79	
amyris oil	16.36	204, 189, **161**, 122, 107	
	16.6	204, 189, 175, **161**, **93**, **59**	
	17.1	204, 189, 161, **93**, **69**	
	18.56	220, 161, 105, 91, 79, 59	
	19.06	222, 204, 189, 161, 133	
	19.44	222, 204, 189, 161, 133	
	20.08	222, 204, 149, 59	
	20.1	222, 164, 149, 59	
	23.87	222, **109**	
benzyl benzoate	23.95	212, 194, 165, 105, 91, 77, 63, 50	
benzyl alcohol	3.43	108, 79, 77	
cedar wood oil	3.25	101, 89, 71, 59, 45	
	11.95	204, 189, 175, 161, 147, 133, 119, 107	
	12.14	204, 189, 161, 147, 133, **119**	
	18.43	222, **150, 95**	cedrol
Clearwood	-	-	-
copaiva balsam	12.06	204, 189, 175, 161, 147, 133, 119, 105, 91, 77	Coelution with cycloseychellene. aromadendrane
	12.75	204, 189, 175, 161, 145, 133, 119, 105, 91, 81, 77	aromadendrane
	14.04	204, 189, 175, 161, 147, 133, 119, 107, 91, 81	
diethyl phthalate	18.1	222, 177, 149	
dioctyl phthalate	39.05	279, 167, 149	
gurjun balsam	3.31	277, 184, 168, 136, 121, 93	
	4.19	136, 132, 121, 105, 93, 79	
	12.06	204, 189, 175, 161, 147, 133, 119, 105, 91, 77	Coelution with cycloseychellene. aromadendrane

	12.73	204	aromadendrane
	14.04	204, 189, 175, 161, 147, 133, 119, 107, 91, 81	
Hercolyn	34.7	289, 243	
	34.85	289, 243	
	35.11	289, 229, 121	
	35.51	281, 163	
	35.68	299, 239, 241	
	35.77	286, 271, 258, 243	
	35.97		
	36.27	299, 239	
	36.89	299, 281, 273	
isobornyl acetate	8.37	154, 136, 121, 108, 95	
methyl benzoate	4.28	136, 105, 77	
paraffin oil	-	-	-
paraffin viscid	-	-	-
pepper oil	3.24	136, 121, 93	Coelution
	3.38	134, 119	Coelution
	3.43	136, 121, 107, 93, 78, 68	Coelution
	3.49	154, 139, 111	
	12.35	204, 133, 93	
propylene glycol	-	-	-
Ricinus oil	31.56	280, 98	
Vetiver oil	17.06	222	Coelution with amyris oil
	20.8	220, **150**	Coelution with compounds from PEO
	21.05	222, **177**	Trace level only detectable
	23.1	220, 202, 189, 159, 150, 131, 119	
	24.67	220, 202, 187, 159, 145, 131, 119, 105	
	24.94	220, 202, 160, 145, 121, 105, 93	
	25.51	**218**, 203, 161, 105, 91	
	25.64	**218, 136**	
	25.98	218, 203, 161, 105, 91	
	26.35	**218, 185**	
Anthemis	6.15	154, 147, 136, 121	

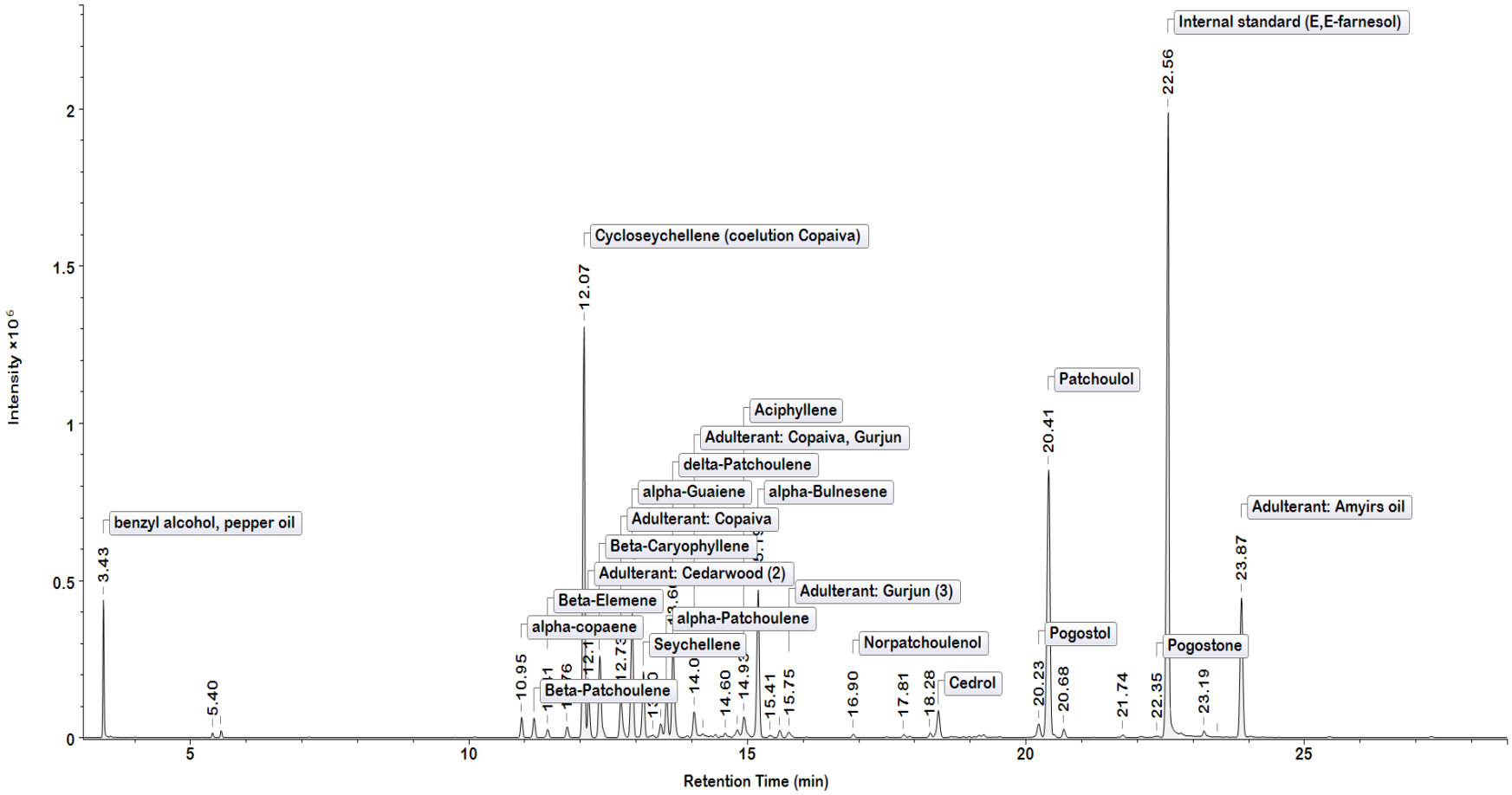

Figure 6.1: TIC of the commercial adulterated sample from Jacob Hooy old. Clearly visible is the adulteration with benzyl alcohol and a possible adulteration with gurjun or copaiva as the cycloseychyllene peak is suspiciously high. Also adulteration with cedar wood as cedrol at 28.28 min can be certainly detected.

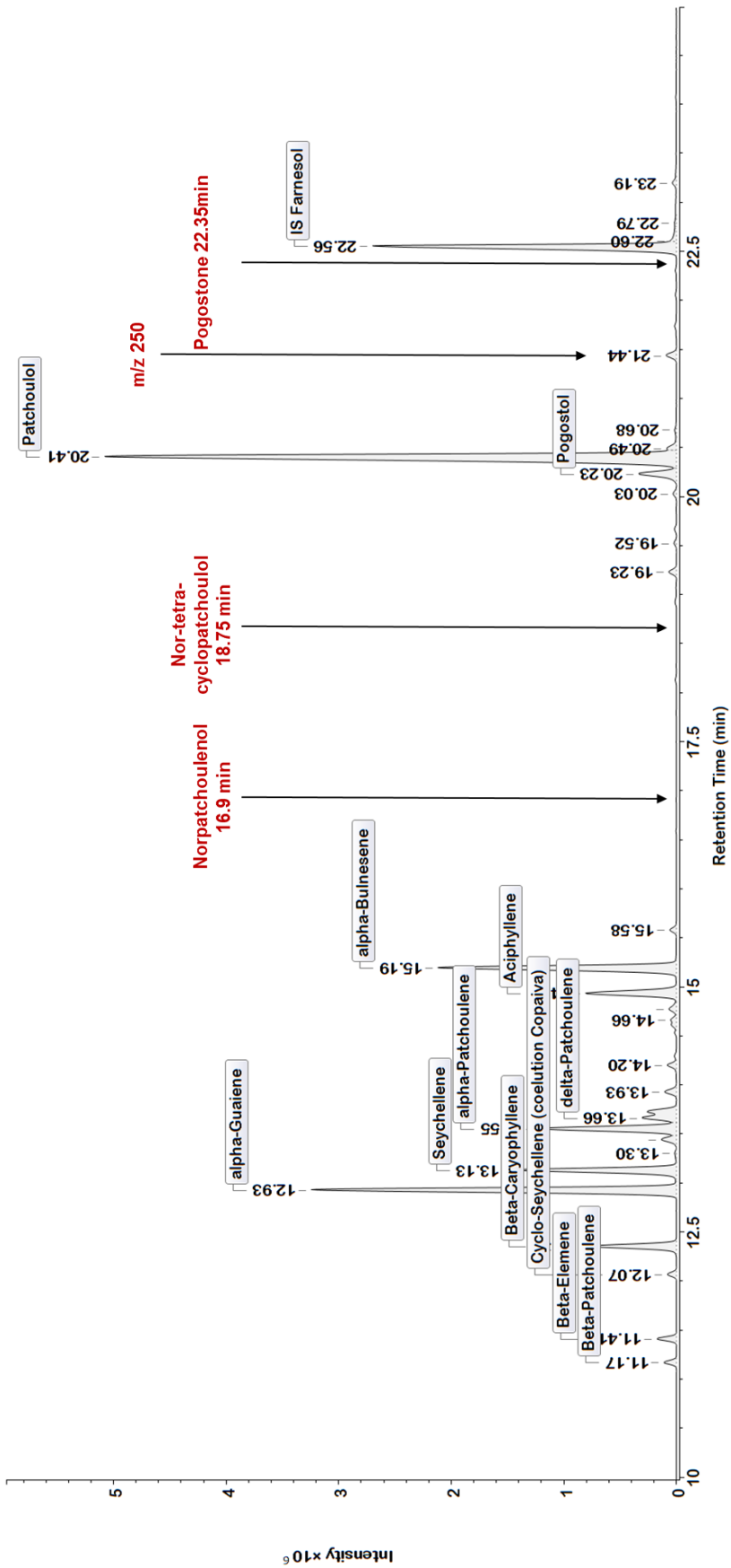

Figure 6.2: TIC of Clearwood. Depicted is the region from 10 min to 25 min were all of the constituents elute. Marked in red at 16.9, 18.75 and 22.35 min are the constituents which are not present in Clearwood. With a peak at 21.44 min and a mass of 250 shows Clearwood an additional distinctive feature compared to PEO.

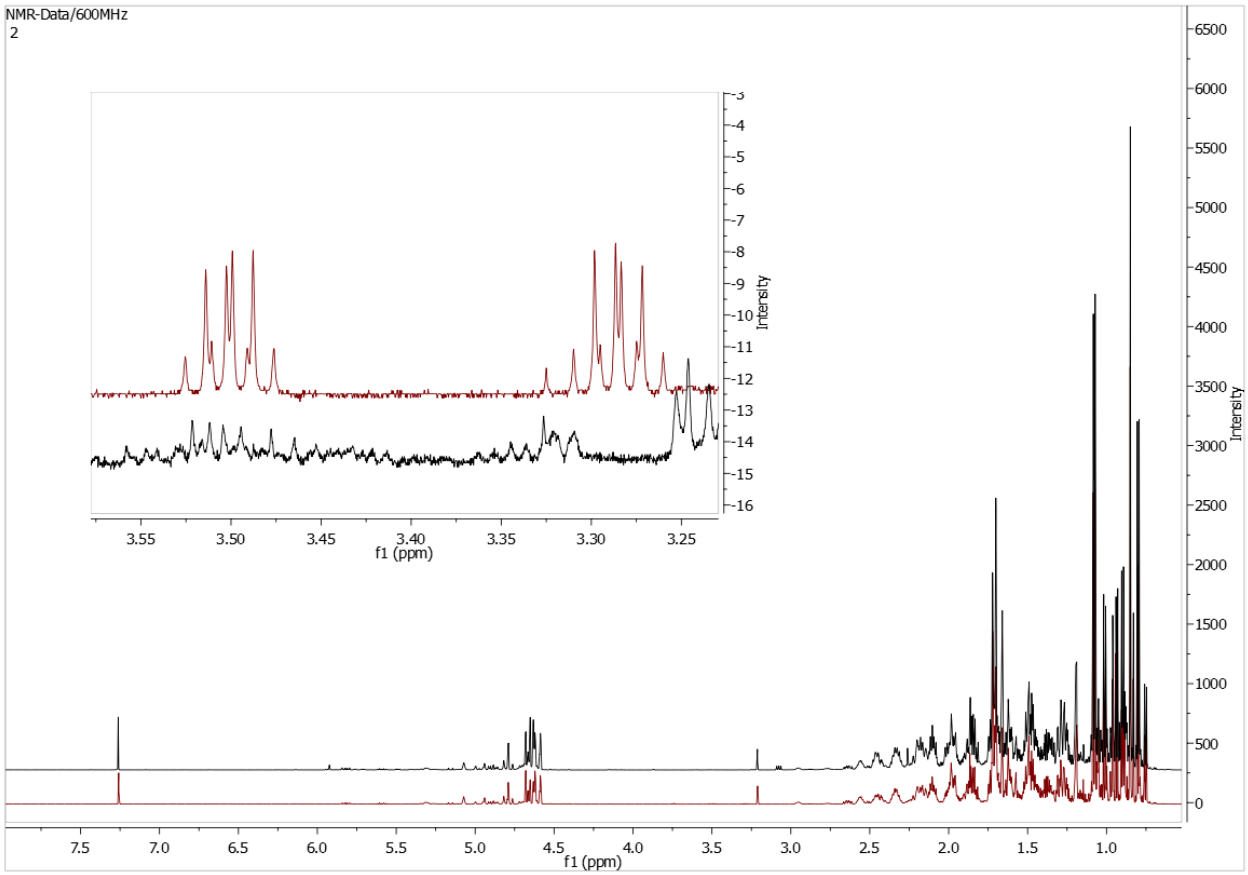

Figure 6.3: ^{1}H 600 MHz NMR of genuine PEO (black) and Clearwood (red). Zoom in to indicate the putative ethylation (CH_3-CH_2-R) signal at 3.28 ppm and 3.5 ppm of the by-product of Clearwood production.

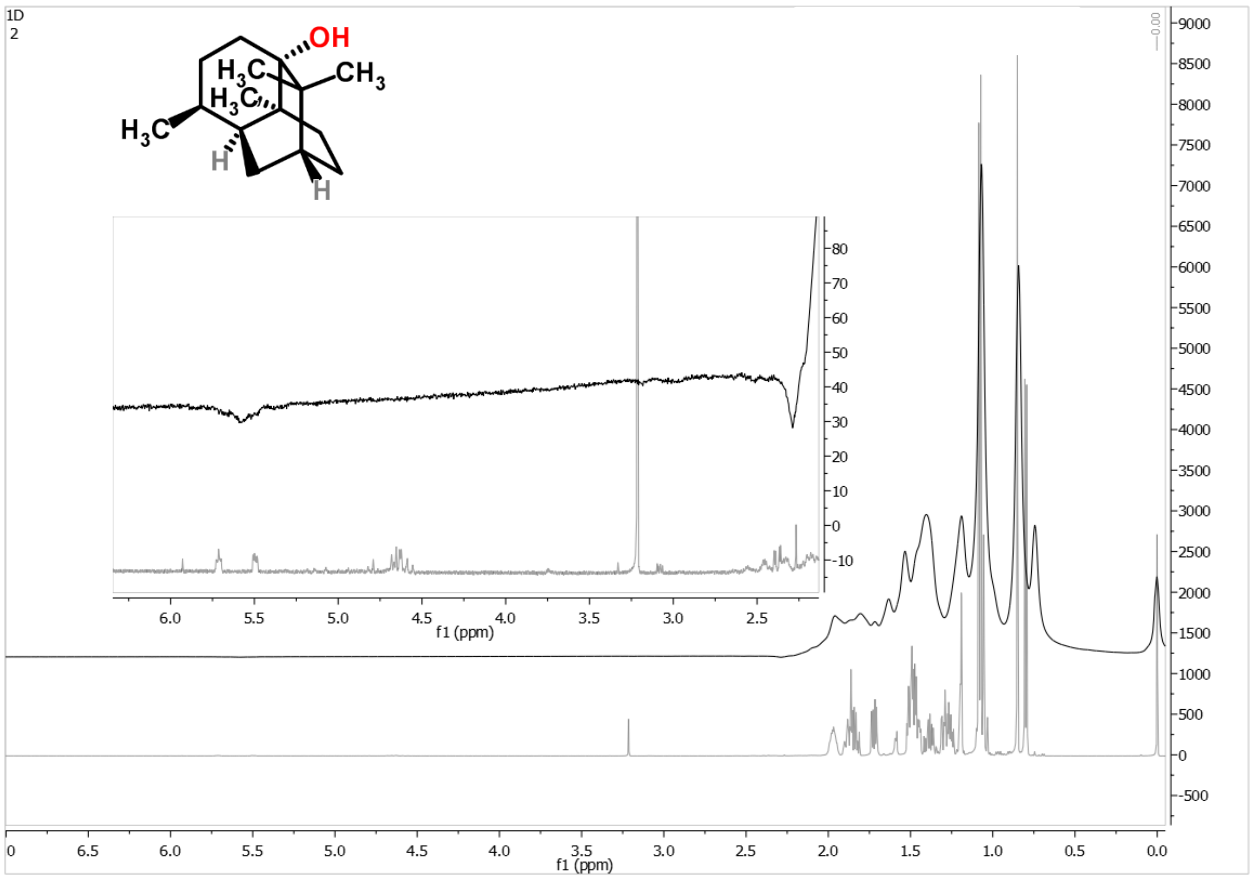

Figure 6.4: Shown is the ^{1}H 60 MHz (black) and the ^{1}H 600 MHz (grey) spectra of patchoulol (structure taken from chemspider.com). Zoom into the region from 2 – 6.5 ppm to indicate the higher resolution of 600 MHz compared to 60 MHz.

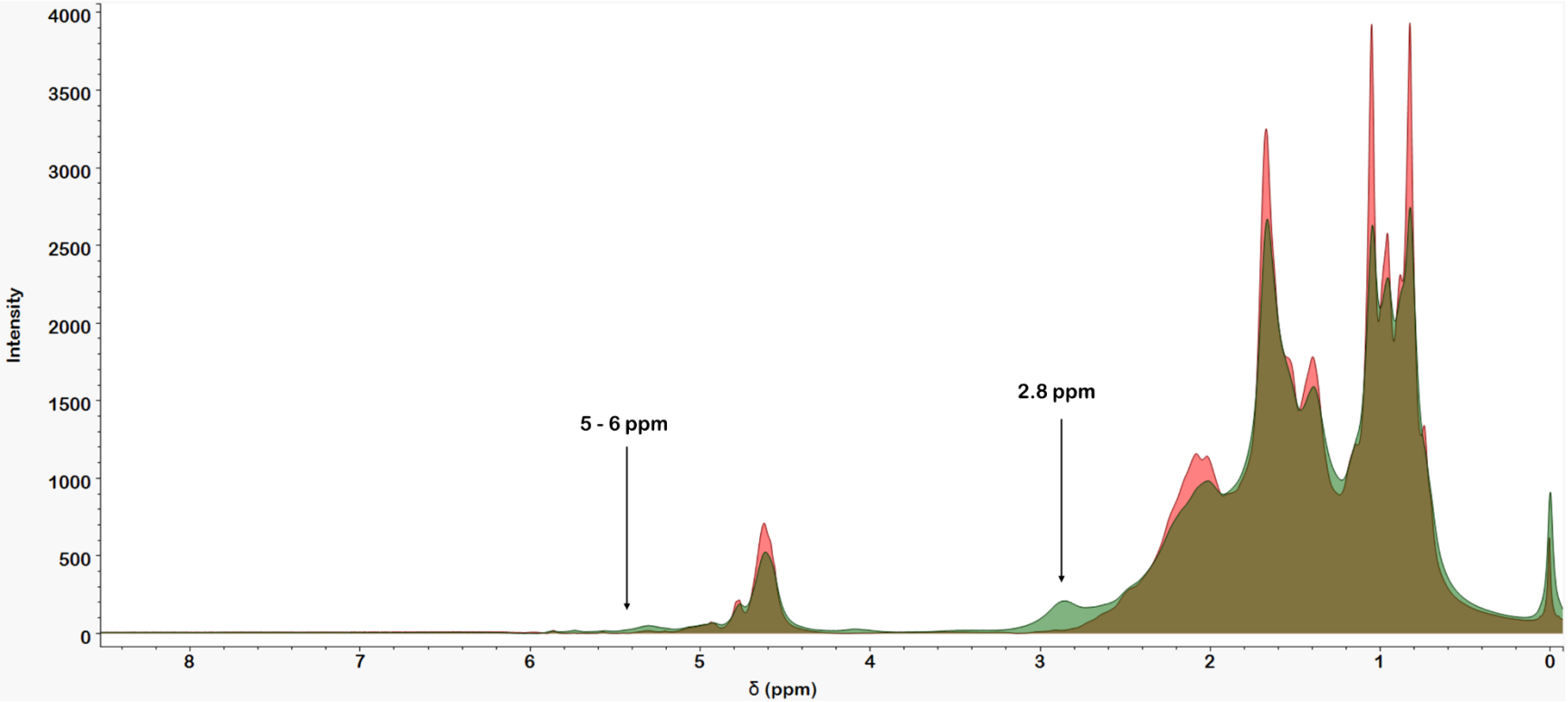

Figure 6.5: In red is the genuine oil of Robertet. Green is a 20% adulteration with vetiver. Cleary visible is the elevated area at 2.8 ppm and slight changes in the area from 5 to 6 ppm.

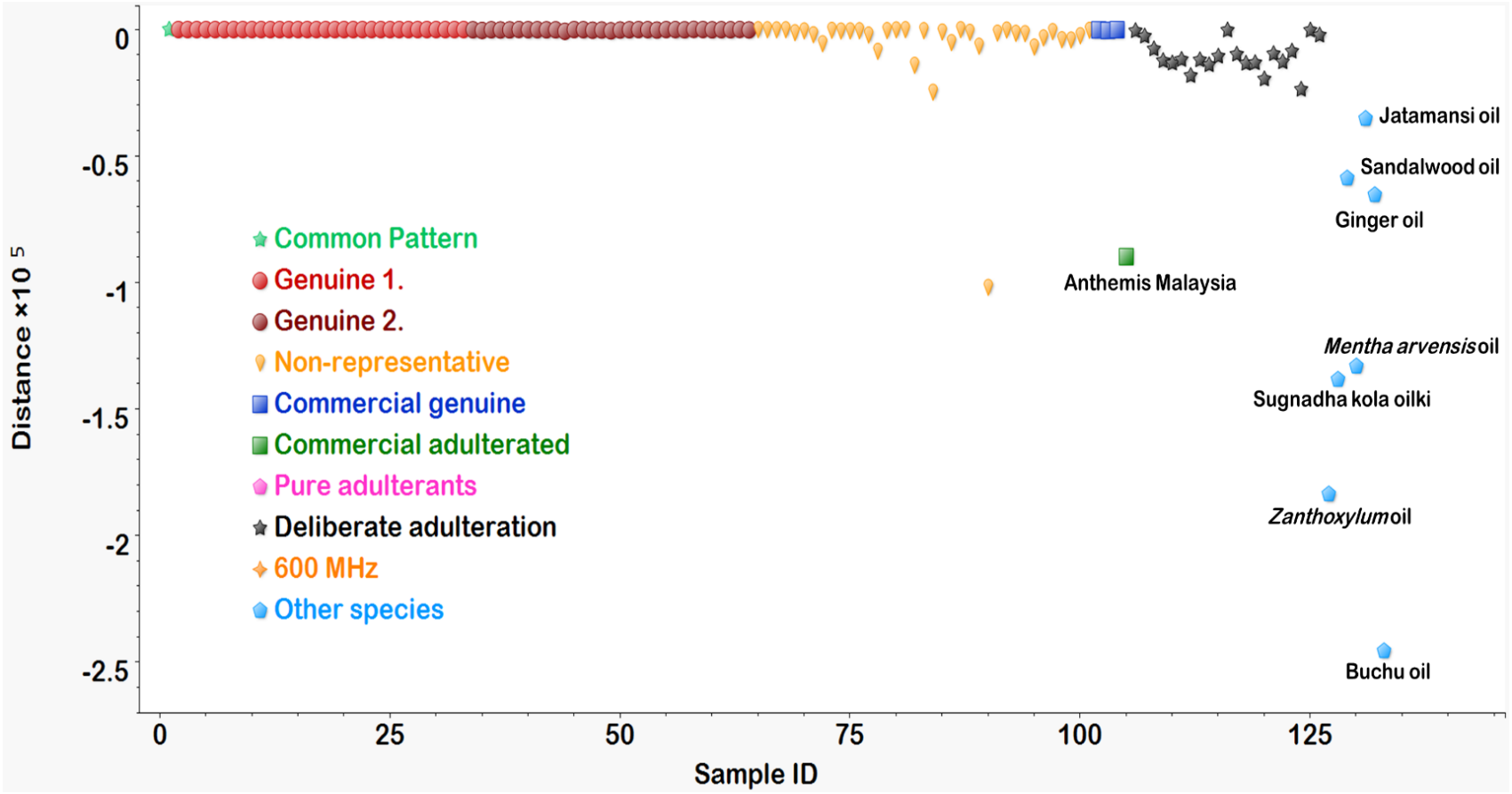

Figure 6.6: Picture to indicate why non-representative samples were sorted out. If samples are within the range and marked as genuine, its most likely that the GC-MS pattern showed good results but NMR with peak broadening. Other essential oils are from the depicted source.

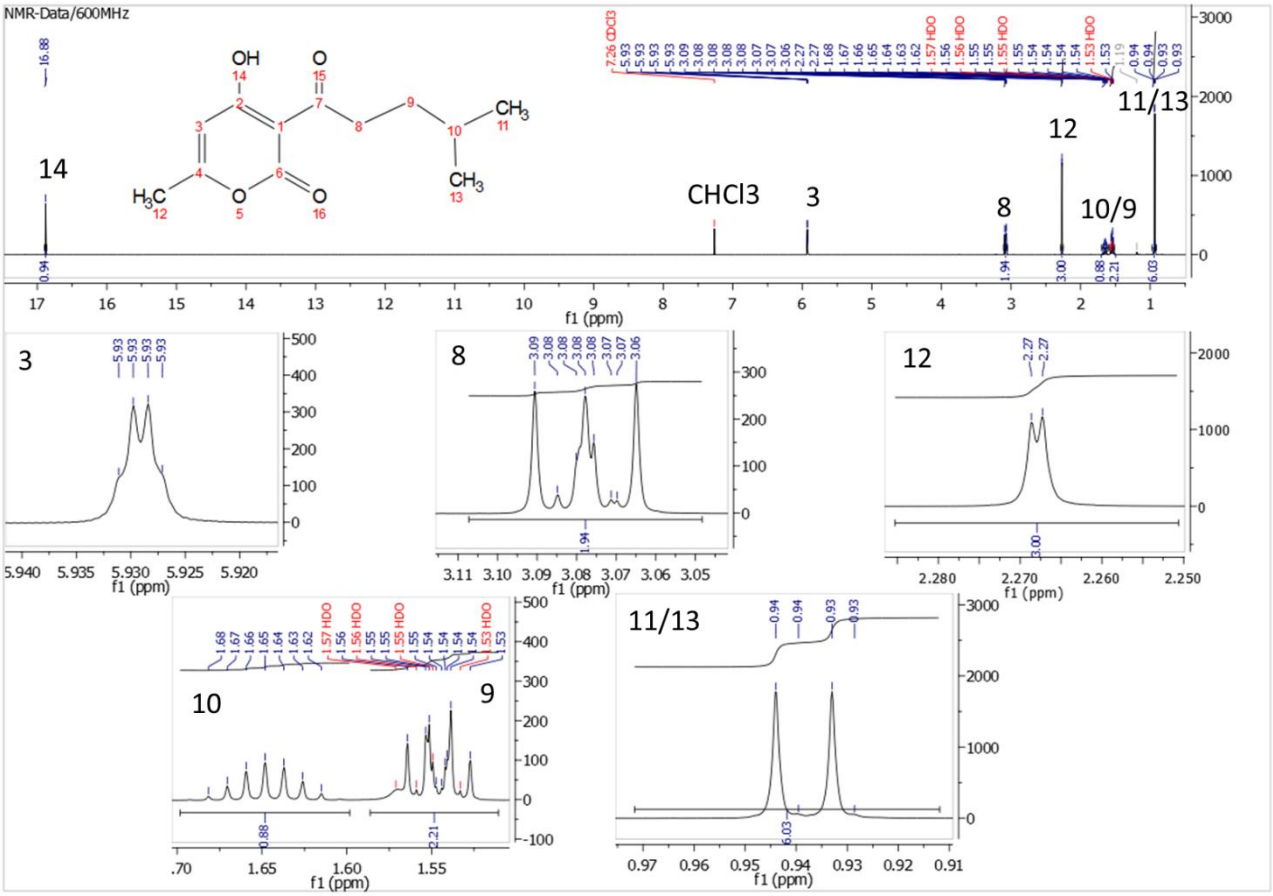

Figure 6.7: ^{1}H 600 MHz spectra of 1% pogostone in $CDCl_3$. Above full spectra and molecular structure including the unusual peak at 16.8 from the Hydroxyl. $CHCl_3$ indicates the peak of residual Chloroform. Not shown is the region <0.5 to avoid TMS peak. Numbers indicate which H's attached to the C/O show the signal. Below a zoom into the different peak regions.
δ 16.88 (1H, s), δ 5.93 (1H, d, J = 0.8 Hz), δ 3.075 (2H, t, J = 7.5 Hz), δ 2.27 (1H, d, J = 0.8 Hz), δ 1.65 (8H, non J = 6.7 Hz), δ 1.54 (3H, q, J = 7.7 Hz), δ 0.935 (1H, d, J = 6.6 Hz).