

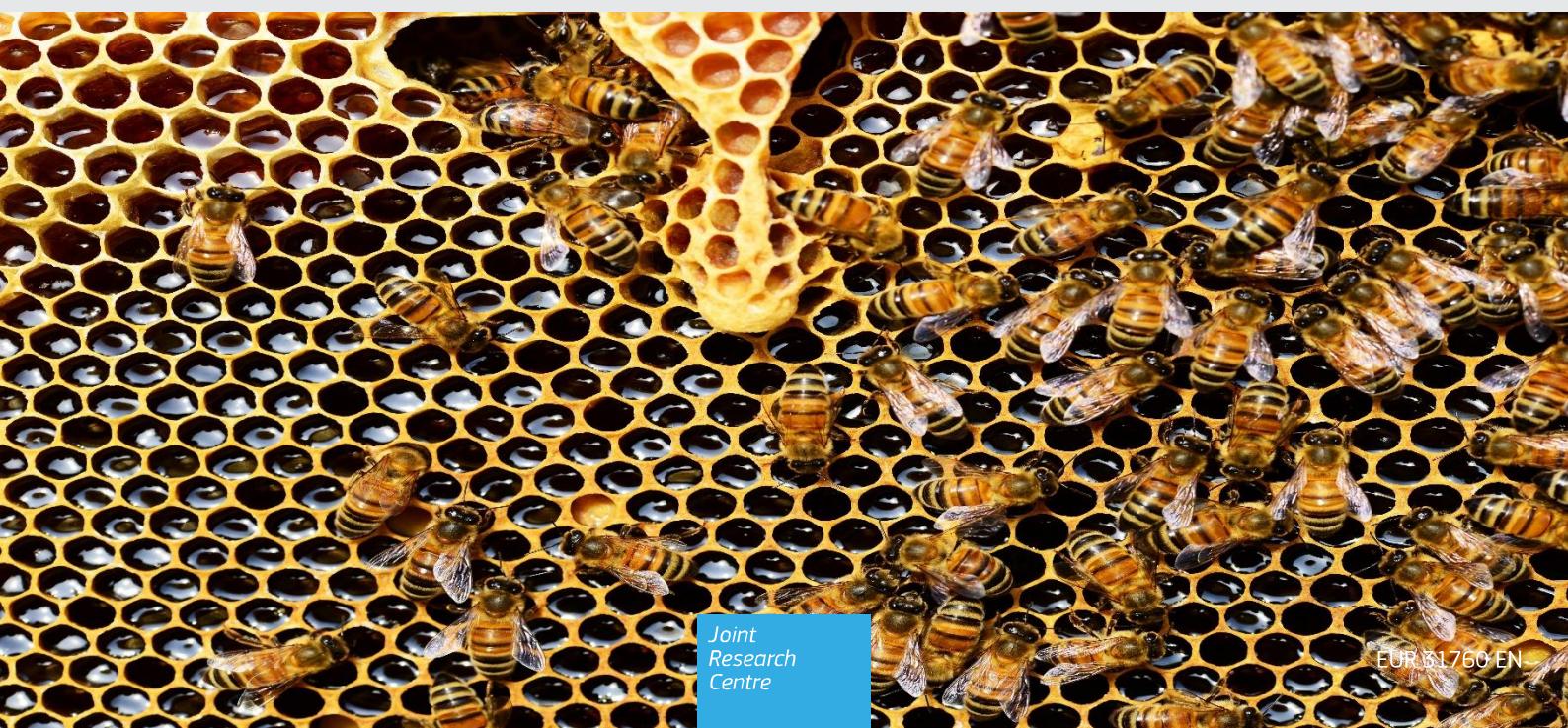


JRC TECHNICAL REPORT

# Analytical methods for the determination of paraffin *n*-alkanes and stearin/stearic acid in beeswax

Araújo, M, Boix Sanfeliu, A, Chatzipanagis, K,  
Ioannou, Th, Karasek, L, Wenzl, Th

2023



This publication is a Technical report by the Joint Research Centre (JRC), the European Commission's science and knowledge service. It aims to provide evidence-based scientific support to the European policymaking process. The contents of this publication do not necessarily reflect the position or opinion of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication. For information on the methodology and quality underlying the data used in this publication for which the source is neither Eurostat nor other Commission services, users should contact the referenced source. The designations employed and the presentation of material on the maps do not imply the expression of any opinion whatsoever on the part of the European Union concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

**Contact information**

Name: Thomas Wenzl

Address: Retieseweg 111, B-2440 Geel

Email: thomas.wenzl@ec.europa.eu

Tel.: +32 14 571320

**EU Science Hub**

<https://joint-research-centre.ec.europa.eu>

JRC135548

EUR 31760 EN

PDF ISBN 978-92-68-09935-3 ISSN 1831-9424 <https://doi.org/10.2760/09730>

Luxembourg: Publications Office of the European Union, 2023

© European Union, 2023



The reuse policy of the European Commission documents is implemented by the Commission Decision 2011/833/EU of 12 December 2011 on the reuse of Commission documents (OJ L 330, 14.12.2011, p. 39). Unless otherwise noted, the reuse of this document is authorised under the Creative Commons Attribution 4.0 International (CC BY 4.0) licence (<https://creativecommons.org/licenses/by/4.0/>). This means that reuse is allowed provided appropriate credit is given and any changes are indicated.

For any use or reproduction of photos or other material that is not owned by the European Union permission must be sought directly from the copyright holders. The European Union does not own the copyright in relation to the following elements:

- Cover page illustration, Image by PollyDot from Pixabay

How to cite this report: Araújo, M, Boix Sanfeliu, A, Chatzipanagis, K, Ioannou, Th, Karasek, L, Wenzl, Th, *Analytical methods for the determination of paraffin n-alkanes and stearin/stearic acid in beeswax*, 2023, EUR 31760 EN, <https://doi.org/10.2760/09730>

## Contents

Contents.....	i
Abstract.....	1
1 Introduction.....	3
2 Samples.....	6
2.1 Test samples.....	6
2.2 Test sample treatment and subsampling.....	6
2.3 Calibration and reference samples for spectroscopic analysis.....	6
3 Infrared spectroscopy based analytical method.....	7
3.1 Analytical method.....	7
3.2 IR spectra of beeswax and adulterants.....	8
3.3 Principal Component Analysis (PCA).....	9
3.4 Partial Least Squares (PLS).....	13
3.5 Transferability study.....	15
4 Chromatography based analytical method.....	17
4.1 Outline of chromatography based analytical method.....	17
4.2 Optimizations of sample preparation.....	18
4.2.1 Solid phase extraction.....	18
4.2.2 Derivatisation of stearin/stearic acid.....	19
4.3 Method validation data.....	21
4.3.1 Linearity.....	21
4.3.2 LOD and LOQ.....	22
4.3.3 Matrix effect.....	22
4.3.4 Selectivity.....	22
4.3.5 Repeatability and Intermediate Precision.....	22
4.4 Typical contents and thresholds.....	24
4.4.1 Paraffin <i>n</i> -alkanes.....	24
4.4.2 Stearin/stearic acid.....	29
4.5 Method transferability study.....	31
5 Conclusions.....	32
References.....	33
List of abbreviations and definitions.....	35
List of figures.....	37
List of tables.....	38
Annexes .....	39
Annex 1. Standard operating procedure for the determination of paraffin and stearic acid in beeswax by Attenuated Total Reflectance Infrared Spectroscopy (ATR-IR).....	39
Annex 2. Standard operating procedure for the determination of paraffin and stearin/stearic acid in beeswax by gas chromatography .....	42
Annex 3. Sample description.....	56

## **Abstract**

Bees are important pollinators. They are essential for agricultural production and have major influence on the biodiversity of plants. Beeswax is after honey the second most important commercial bee product with a global market size of more than half a billion Euros. Beeswax is secreted in small amounts from special wax glands of young honeybees. Beeswax is used as a food additive, in cosmetics, in pharmaceutical, many other products such as candles, or leather protection creams, as well as in beekeeping for the production of foundations of honeycombs. It is more expensive than other natural waxes, which makes it susceptible to fraud. Adulteration of beeswax might not only have economic consequences; consumer protection and animal health aspects play also a role. Dilution or replacement of beeswax by cheaper products such as paraffin or stearin/stearic acid were reported. In support of the Commission Farm-to-Fork Communication, analytical methods based on attenuated total reflectance infrared spectroscopy and on gas chromatography were developed for the determination of paraffin n-alkanes and of stearin/stearic acid in beeswax.

***Authors***

Name	Contribution
Márcia Araújo	E
Ana Boix Sanfeliu	C
Konstantinos Chatzipanagis	C, E, R
Theodora Ioannou	E
Lubomir Karasek	E
Thomas Wenzl	C, R

C: conception, E: experiments, R: reporting

## 1 Introduction

Beeswax is produced by honey bees such as the western honey bee (*Apis mellifera*). They contain a special gland, which secretes liquid beeswax. The liquid solidifies in contact with air and forms small scales (Cassier & Lensky, 1995). Bees use the beeswax scales for the production of the hexagonal cells and lids of honeycombs, in which they store honey and pollen. Also the bee brood is raised in honeycombs.

Beeswax is composed of a variety of substances belonging to different classes of chemicals. Major constituents are alkanes, alkenes, free fatty acids, monoesters, diesters, and hydroxymonoesters. Unhydrolyzed beeswax derived from combs consists of approximately 67 % of esters, 14 % hydrocarbons, 12 % free fatty acids, and 6 % other substances (Tulloch, 1980). The specific composition of the beeswax depends of the bee species, the age of the bee, and environmental factors (Hepburn, Pirk, & Duangphakdee, 2014). The different processes used for beeswax handling in combination with changes associated with aging of beeswax can also effect the chemical composition of authentic beeswax. Furthermore, beeswax composition shows considerable differentiations depending on its origin. Beeswax deriving from *Apis mellifera*, the bee species raised in Europe, has similar properties as the African beeswax, with small differences in the ratio of specific compounds. The composition of Asian beeswax, produced by the eastern honey bee (*Apis cerana*) is less complex containing fewer compound than beeswax of European or African origin (Tinto, Elufioye, & Roach, 2017). Another property that shows high diversity is the colour of beeswax. Newly made beeswax has white colour which changes overtime to yellow and brown tones, which are caused by bees' excretions and propolis - pollen pigments. Was et al. found differences in *n*-alkane contents of light and dark coloured beeswax (Waś, Szczesna, & Rybak-Chmielewska, 2014b).

Beeswax is used for many purposes. The global economic value is estimated to more than half a billion Euros. The online platform "Tridge.com", which collects and compiles global trade data of food and agricultural products, indicates for beeswax (tariff code 152190: beeswax, other insect waxes and spermaceti, whether or not refined or coloured) an annual export volume of about 120 million US dollar. Most of the beeswax is used in cosmetics and in the pharmaceutical sector. Smaller quantities are used for the production of amongst others candles, waterproofing agents, lubricants, and care products for leather and furniture. The apicultural sector uses and recycles beeswax into foundations for honey combs.

Both white and yellow beeswaxes are authorised food additives (E 901) for which Commission Regulation (EU) No 231/2012 sets purity criteria. Also the European Pharmacopoeia lists purity criteria for beeswax, (Council of Europe, 2020; European Union, 2008). No purity criteria exists for beeswaxes intended for technical purposes in the apicultural sector.

The magnitude of beeswax adulteration was recently investigated in a nationwide survey in Belgium (El Agrebi et al., 2021). The survey revealed that about 9 % and about 33 % of beeswax for beekeeping and commercial beeswax products respectively were adulterated with paraffin and stearin/stearic acid. The beeswax for beekeeping was primarily adulterated with stearin/stearic acid, which showed to have adverse effects on the development of the bee brood (Reybroeck, 2017).

The European Food Safety Authority (EFSA) was requested by the Commission to assess risks associated with the adulteration of beeswax with paraffin and/or stearin/stearic acid (European Food Safety Authority, 2020). EFSA itemised the task into the assessments of effects of the exposure to adulterated beeswax on bee health and on human health. The human consumption of stearin and stearic acid would not be of concern provided that the products are of food grade purity. Of higher concern rated was the consumption of beeswax adulterated with paraffin, as EFSA assumed that paraffin applied for the adulteration of beeswax would not comply with the highest purity criteria and might thus contain hazardous constituents. The impact of beeswax adulteration on bee health was more difficult to assess as relevant toxicological data are lacking to a big extend. However, EFSA referred to studies conducted in France and Belgium, which showed increased mortality rates of the bee brood under exposure to low levels of stearic acid and palmitic acid (Reybroeck, 2017). Castro et al investigated the effect of beeswax foundations adulterated with up to 40 % of paraffin on the tilling of honeycombs and the viability of *Apis mellifera* pups (Castro, Medici, Sarlo, & Eguaras, 2010). Without providing further evidence, they reasoned the increased viability rate of the pups exposed to high levels of paraffin with the dilution of potential contaminants contained in the pure beeswax by the added paraffin.

EFSA recommended applying at least two physico-chemical parameters complemented by analytical methods based on gas chromatography with flame ionisation or mass spectrometric detection, or attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) for the detection and

quantification of adulterations of beeswax with paraffin and/or stearin/stearic acid. The limits of detection of the analytical methods should be below 5 % of mass.

DG SANTE commissioned via the administrative arrangement "Technical support to combat agri-food fraud" amongst others the optimisation and validation of appropriate analytical methods to detect the adulteration of beeswax with paraffin and/or stearin with a view to assist Member States authorities in shaping their controls. Both a screening method based on vibrational spectroscopy and a confirmatory method based on chromatography had to be developed and single-laboratory validated. The required diagnostic sensitivity of the chromatographic method was specified as the capability to detect 5 % adulterant in pure beeswax.

Paraffin or paraffinic hydrocarbons are straight-chain or branched saturated hydrocarbons, with carbon and hydrogen atom contents following the general formula  $C_nH_{2n+2}$ . Besides *n*-alkanes, paraffin contains varying amounts of iso-alkanes, and cycloalkanes (naphthenes). Paraffin is usually derived from petroleum or coal. It may be liquid or solid at room temperature. The latter is a white, odourless, macro- or microcrystalline product. It consists of a mixture of hydrocarbons with chain lengths in the range of about 20 to 40 carbon atoms. The melting point of solid paraffin is in the range of 46 °C to 68 °C (Freund & Mózes, 1982).

Stearin is the synonym for glyceryl tristearate, an odourless, white powder, which is obtained as byproduct from the processing of animal fat. The term stearin is also used to denote the solid fraction of plant oils, e.g. palm stearin. On this note, stearin refers to the technological properties of the oil fraction rather than to chemical composition, as palm stearin contains in the glycerides both palmitic acid and stearic acid (Idris Nor & Mat Sahri, 2007). Stearic acid (octadecanoic acid) is a chemically well-defined waxy solid. Both stearin and stearic acid might be used for the hardening of waxes, e.g. in candle production.

Beeswax contains naturally alkanes and alkenes. The most abundant hydrocarbon compounds in pure beeswax are odd-numbered straight hydrocarbons and especially *n*-alkanes with 25 to 33 carbon atoms. The presence of elevated levels of hydrocarbons with an even number of carbon atoms possibly indicate the substitution of beeswax with paraffin (Juan José Jiménez, Bernal, del Nozal, Toribio, & Bernal, 2007; M. Maia & Nunes, 2013; Waś, Szczesna, & Rybak-Chmielewska, 2016).

Scientific literature is rich on papers dealing with the elucidation of the composition of beeswax. There is less information on analytical methods for the identification of adulterations of beeswax. Gas chromatography or infrared spectroscopy were applied in recent years for this purpose. Both gas chromatography with flame ionisation detection and gas chromatography mass spectrometry were suitable for the determination of adulterations with paraffin (Chen et al., 2012; Juan José Jiménez, Bernal, del Nozal, Martín, & Toribio, 2009; Juan José Jiménez et al., 2007; M. Maia & Nunes, 2013; Serra Bonvehi & Orantes Bermejo, 2012; Špaldoňová, Havelcová, Lapčák, Machovič, & Titěra, 2021; Waś, Szczesna, & Rybak-Chmielewska, 2014a; Waś et al., 2016). Gas chromatography with flame ionisation detection has the advantage of a wide linear range and similar response factors for analytes with similar structure, e.g. *n*-alkanes with similar number of carbon atoms. Gas chromatography mass spectrometry offers increased selectivity compared to flame ionisation detection and provides information on the identity of the measured substance.

Different routes were applied within the rubric of gas chromatography based methods for the identification of adulteration of beeswax with paraffin. Jiménez et al. proposed to use concentration guide values and relative concentration ratios between adjacent odd and even numbered *n*-alkanes for discriminating pure and adulterated beeswax (Juan José Jiménez et al., 2007). Jiménez and co-workers found also that the relative concentration of several palmitates were negatively correlated with the concentrations of even numbered *n*-alkanes, which may be explained by the dilution of wax esters by paraffin (Juan José Jiménez et al., 2009). This dilution is not exclusive to palmitates. Hence the monitoring of different minor beeswax constituents offers additional possibilities to identify the adulteration of beeswax with foreign substances (Juan José Jiménez et al., 2009; Serra Bonvehi & Orantes Bermejo, 2012). The direct comparison of individual *n*-alkanes, or groups of *n*-alkanes with the respective values with results obtained for virgin beeswax is also suitable (Waś et al., 2016). The reported limits of detection of adulterations with paraffin, expressed as percentage of mass, were for all methods similar. They were in the range of 3 % to 5 % of sample weight.

Fewer papers deal with the adulteration of beeswax with stearin/stearic acid (Juan José Jiménez et al., 2009; Serra Bonvehi & Orantes Bermejo, 2012; Svečnjak et al., 2019). However, there is consensus that pure beeswax contains stearic acid at very low concentration at maximum (Svečnjak et al., 2021). Free

stearic acid or stearic acid bound in glycerides are determined by gas chromatography after derivatisation to methyl esters. There is rich literature on the determination of free and bound fatty acids in different kinds of food. Depending of the applied derivatisation reaction, it is possible to discriminate between free and bound stearic acid, or to determine them together.

From a spectroscopy point of view, Fourier transform infrared spectroscopy (FTIR) with an attenuated total reflection (ATR) accessory has been previously employed to detect adulteration in beeswax with approximately or below 5% for various adulterants such as hydrocarbon waxes, beef tallow and stearic acid (Miguel Maia, Barros, & Nunes, 2013). In another study, an analytical method based on ATR data was developed to determine beeswax adulteration with paraffin, beef tallow, stearic acid and carnauba wax with a detection limit of <3% (Svečnjak et al., 2015). In addition, it has been reported that stearic acid, palmitic acid and commercially available stearin, exhibit very similar infrared absorption features and thus, the spectral regions characteristic for stearic acid can be also used to detect palmitic acid and stearin in beeswax (Svečnjak et al., 2019). Further progress was achieved by Tanner and Lichtenberg-Kraag who demonstrated that multicomponent adulteration with up to five adulterants (paraffin, stearic acid, tallow, carnauba wax and candelilla wax) could be detected as accurately as single component adulteration (Tanner & Lichtenberg-Kraag, 2019). Apart from investigations on beeswax samples received from random sources, systematic studies have been also performed to identify percentages of paraffin and stearic acid adulteration on a national level (Belgium), showing that commercial beeswax was more prone to adulteration compared to beekeepers beeswax samples (El Agrebi et al., 2021). Based on the above mentioned reports, it is possible to detect beeswax adulteration of less than 3% of these adulterants and their combinations by ATR-FTIR spectroscopy.

In the present study, ATR-FTIR spectroscopy followed by advanced statistical techniques is applied as a screening method to determine the adulteration of beeswax by paraffin and stearic acid. To the best of our knowledge, this is the first study where a fully comprehensive statistical evaluation is performed by combining PCA with PLS regression to explore beeswax adulteration and estimate concentrations of adulterants, respectively. Gas chromatography based analytical methods were developed in order to confirm the results of the screening method, and to allow an estimation of the levels of potential adulteration of beeswax with paraffin respectively stearin/stearic acid.

## 2 Samples

### 2.1 Test samples

More than 100 test samples were retrieved from different sources for the purpose of this work. They comprised beeswax from beekeepers (entire honeycombs), and different commercial sources (pearls, blocks, candles, foundation sheets). Among them were 32 honeycomb samples which still contained honey. After hot water extraction of the honey, they were separated into beeswax from the foundation and beeswax from the sidewalls. The latter were used to establish baseline levels for the contents of paraffin and of stearic acid, as it may be assumed that the sidewalls, which were built by the bees and which were not subjected to any other manipulation, contained only typical background levels of the adulterants under investigation. The beeswax samples were sourced from different countries in order to consider in the studies the potential effect of geographical origin. A description of the test samples is given in Annex 3.

### 2.2 Test sample treatment and subsampling

All test samples were kept from light protected at room temperature. Test portions of about 50 mg each were taken from different parts of the respective test sample. This sample amount is at least ten times higher than the amounts used in literature. However the larger amount has the advantage that weighing is more precise and that the analytical results are less affected by potential sample inhomogeneity. Each test sample was then analysed at least in duplicate.

Only the honeycomb samples still containing honey were processed before subsampling. A portion of the honeycomb was extracted in glass beakers with warm deionised water and rinsed with deionised water after completeness of extraction. Afterwards, the honeycombs were dried in a drying cabinet at 40°C. The beeswax of the sidewalls of these honeycombs was separated from the foundation plate by means of a hot wire cutter. Both types of waxes were analysed in duplicate.

### 2.3 Calibration and reference samples for spectroscopic analysis

Calibration samples intended for infrared investigation were prepared by spiking native beeswax with increasing amounts of paraffin or stearic acid as seen in **Table 1**. Reference samples are paraffin (denoted as samples 6 and 53), stearic acid (denoted as samples 7 and 52), tristearin (Tr) and beef fat (BF). It is noted that sample 6 was used for paraffin calibration and sample 7 for stearic acid calibration.

The beeswax matrix used for the spiking originated from mixing three different beeswax samples that were seen as non-adulterated.

**Table 1.** Calibration samples for paraffin and stearic acid with nominal concentrations.

Paraffin calibration samples	C3P1	C3P2	C3P3	C3P4	C3P5	C3P6	C3P7	C3P8
Paraffin concentration (% w/w)	0.00	5.02	9.69	15.10	20.07	25.15	49.69	100
Stearic acid calibration samples	C3A1	C3A2	C3A3	C3A4	C3A5	C3A6	C3A7	C3A8
Stearic acid concentration (% w/w)	0.00	0.68	1.14	4.90	14.89	25.65	50.64	100

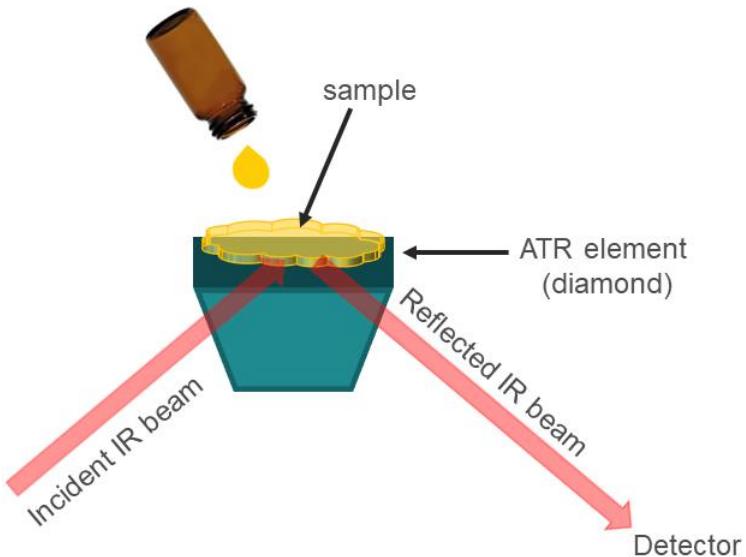
### 3 Infrared spectroscopy based analytical method

#### 3.1 Analytical method

In terms of infrared spectroscopy, an Alpha II compact Fourier-Transform Infra-Red (FTIR) spectrometer (Bruker, Germany) coupled with a platinum attenuated total reflectance (ATR) module containing diamond crystal as the ATR element located on a heating stage and a temperature controlled DLaTGS-detector was used to conduct measurements in the range of  $400 - 4000 \text{ cm}^{-1}$  using 24 scans at  $4 \text{ cm}^{-1}$  spectral resolution. In order to perform spectral acquisition in the liquid state, the samples were heated at  $85^\circ\text{C}$  on a hotplate to achieve complete melting and a drop of the melt sample was uniformly applied on the surface of the diamond crystal, which was also heated at the same temperature in order to prevent specimen solidification during measurement. A background spectrum was recorded prior to each sample measurement and a blank spectrum was subsequently taken after cleaning the crystal to ensure that the cleaning process was successful before the next measurement. Spectra were recorded in duplicates for each sample. Details on the analytical procedure are given in Annex 1.

**Figure 1** is a schematic illustration of the ATR experiment on beeswax samples. After sample deposition on the heated ATR element, an incoming beam is travelling through the diamond crystal and penetrates the specimen. The penetration depth is only a few microns creating an evanescent wave within the sample and then the beam is totally reflected back. Total reflection is the result of the IR beam travelling from a medium of higher refractive index (diamond) to another medium with lower refractive index (sample). The reflected beam is directed out of the diamond crystal, towards a detector. The relative intensity difference between the incident and reflected beam is caused by sample absorbance.

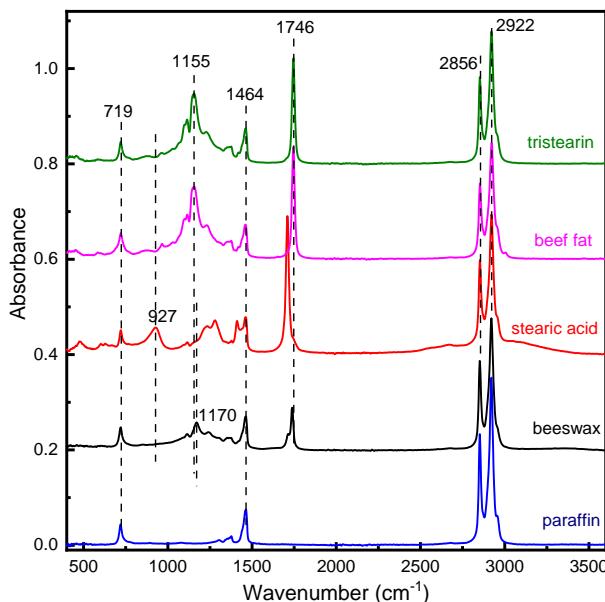
**Figure 1.** Schematic representation of a single bounce ATR experiment on beeswax.



### 3.2 IR spectra of beeswax and adulterants

The IR spectra of beeswax, stearic acid, paraffin, tri-stearin and beef fat samples are illustrated **Figure 2**.

**Figure 2.** IR spectra of paraffin, beeswax, stearic acid, beef fat samples and tri-stearin. Spectra (except paraffin) are shifted for clarity.



This figure shows that the five chemical substances exhibit IR absorption in the spectral ranges  $400 - 1800\text{ cm}^{-1}$  and  $2800 - 3000\text{ cm}^{-1}$ . On one hand, the  $400 - 1800\text{ cm}^{-1}$  region shows significant variations in the number, location and relative intensities of the bands among the substances, except in the case of tri-stearin and beef fat whose spectra are almost identical. On the other hand, all substances share a very similar strong doublet band in the high frequency region at  $2800 - 3000\text{ cm}^{-1}$  that is assigned to  $\text{CH}_2$  vibrations (Jones, McKay, & Sinclair, 1952; Kitagawa, Sugai, & Kummerow, 1962; Muscat, Tobin, Guo, & Adhikari, 2014; Socrates, 2004; Špaldoňová et al., 2021). A detailed assignment of the main IR absorption bands seen for these substances is provided in **Table 2**.

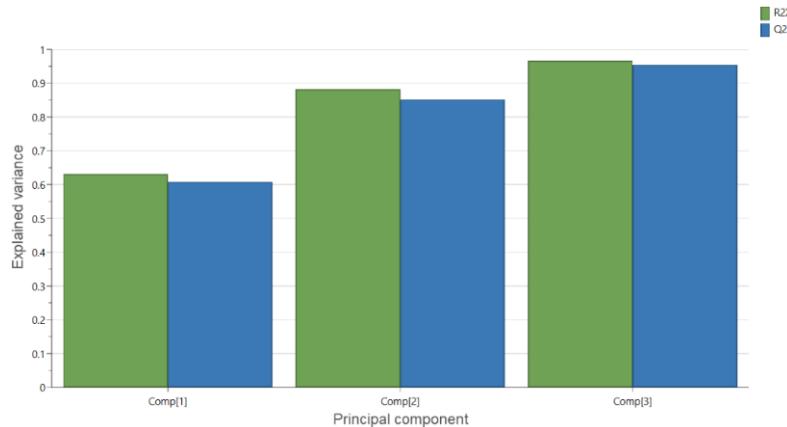
**Table 2.** Vibrational assignments of the main bands present in the IR spectra.

Band assignment	Wavenumber ( $\text{cm}^{-1}$ )
$\text{CH}_2$ rocking mode (hydrocarbons)	719
$\text{C=O}$ stretching and $\text{C-H}$ bending vibrations (esters)	1170
$\text{CH}_2$ scissor deformation (hydrocarbons)	1464
$\text{C=O}$ stretching vibrations (esters, free fatty acids)	1746
$\text{CH}_2$ symmetric stretching	2856
$\text{CH}_2$ asymmetric stretching	2922

### 3.3 Principal Component Analysis (PCA)

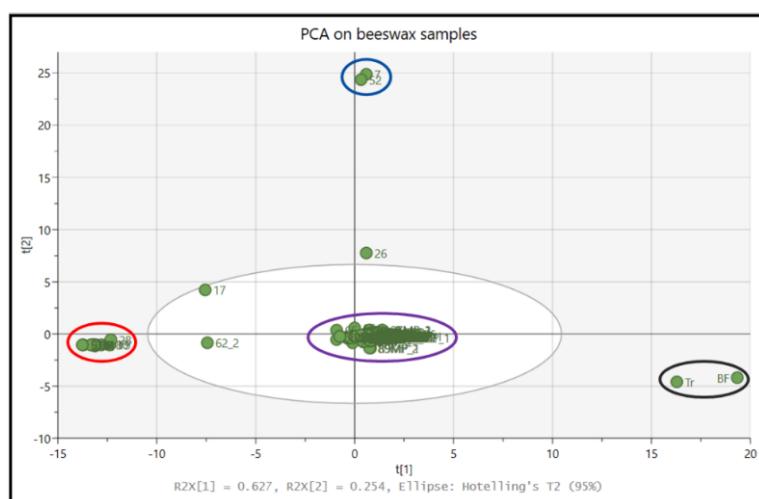
Prior to conducting PCA using the SIMCA 17 software (Umetrics), the raw IR spectra were pre-processed using mean-centering and standard normal variate (SNV) and three principal components were found to explain ~ 96 % of the total variance, as depicted in **Figure 3**. Additional components show no further contribution in the explained variance thus they are excluded from our analysis. The “leave-out” cross validation was used during model fitting.

**Figure 3.** Contribution of various principal components in explained variance.



**Figure 4** shows the scores plot of the first two principal components ( $t[2]$  vs  $t[1]$ ) for the entire set of test and reference samples, explaining ~ 88 % of the sample variance.

**Figure 4.** PCA scores plot of test and reference samples. Reference stearic acid samples (samples 7 and 52) are shown in the blue circle, reference paraffin (samples 6 and 53) and highly paraffin adulterated beeswax samples are shown in the red circle, pure beeswax samples are included in the purple circle and tri-stearin (Tr)/beef fat (BF) reference samples are included in the black circle.

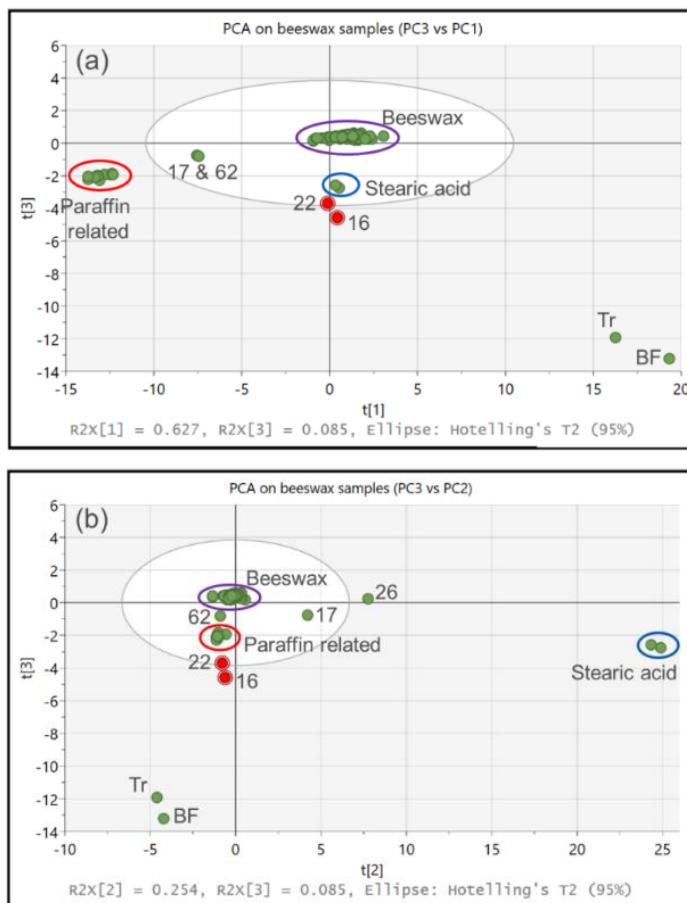


At the top of t[2] the two reference samples of stearic acid are located (blue circle), whereas at the very left of t[1] there are the two reference paraffin samples and a number of other beeswax samples that could be evidently seen as largely adulterated with paraffin. The middle part of t[1] contains a big cluster of beeswax

samples (purple circle) that may be considered as 'pure' due to their distance to the studied adulterants, while reference Tr and BF samples (black circle) are situated at the right part of  $t[1]$ . It is interesting to note that samples 17, 26 and 62 do not belong to any of the four clusters, denoting partial adulteration by one or more adulterants. In fact, sample 26 seems to be adulterated with stearic acid, sample 62 with paraffin and sample 17 seems to contain a mixture of paraffin/stearic acid.

Moreover, evaluation of the third principal component against the first two components demonstrates an additional 9% contribution in the explained variance, as shown in **Figure 5**.

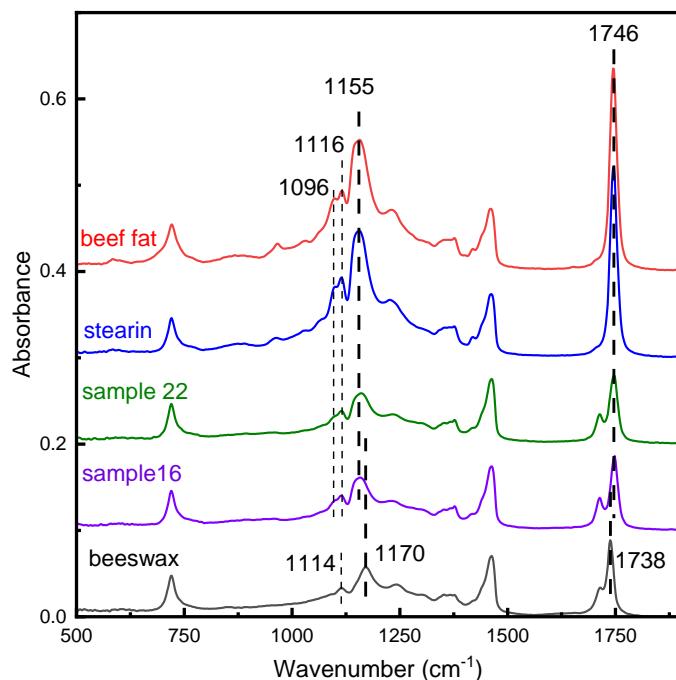
**Figure 5. (a)**. PCA scores plot ( $t_3$  vs  $t_1$ ) of test and reference samples. **(b)**. PCA scores plot ( $t_3$  vs  $t_2$ ) of test and reference samples.



According to these scores plots, samples 16 and 22 appear somehow different from the pure beeswax samples due to their separation from the tight cluster. In particular, the two samples are projected between the pure beeswax cluster and the two reference samples of tri-stearin and beef fat, which indicates that samples 16 and 22 may contain amounts of the latter substances.

**Figure 6** provides a comparison of the untreated IR spectra of tri-stearin, beef fat, pure beeswax, samples 16 and 22. Two spectral features located at  $1155$  and  $1746\text{ cm}^{-1}$  ( $\text{C=O}$  stretching vibrations) are observed in samples 16, 22, tri-stearin and beef fat, whereas the same features in beeswax are found at  $1170$  and  $1738\text{ cm}^{-1}$ , respectively. Furthermore, samples 16 and 22 demonstrate a doublet at  $1096$  and  $1116\text{ cm}^{-1}$  that is also present in tri-stearin and beef fat, whereas the spectrum of beeswax exhibits a single feature at  $1114\text{ cm}^{-1}$ .

**Figure 6.** IR spectra of beeswax, beef fat, tri-stearin and samples 16, 22. Spectra are shifted for clarity.

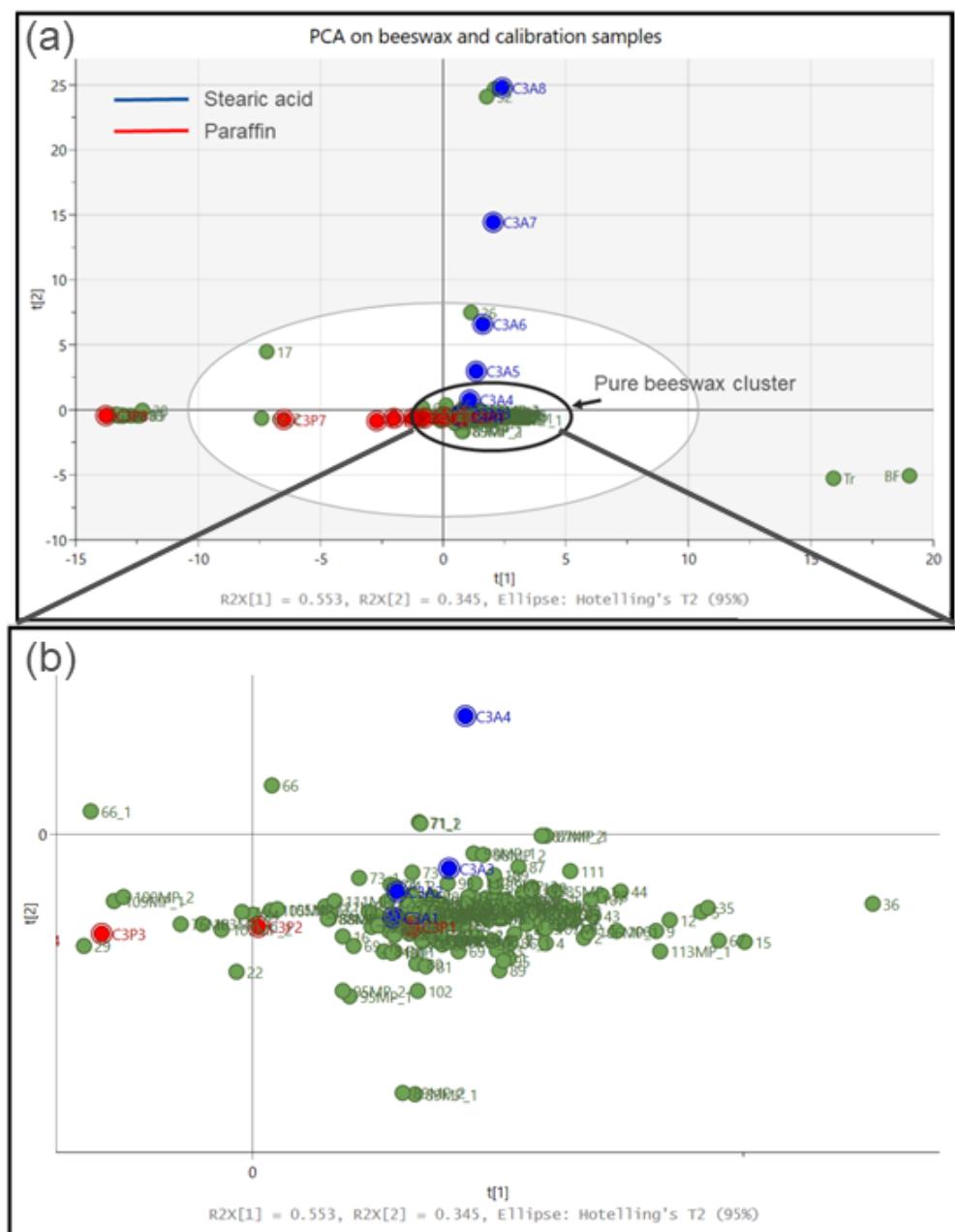


Therefore, visual inspection of the untreated IR spectra seems to support the PCA findings using the third component suggesting that samples 16 and 22 may contain amount of tri-stearin and/or beef fat to a certain extent, in addition to paraffin and/or stearic acid. This assumption was tested and verified by performing a comparison between experimental and calculated spectra computed from reference samples (beeswax, paraffin, stearin, stearic acid) with appropriate weighting factors. Calculations indicate that these two beeswax samples seem to contain moderate amounts of paraffin (~ 20 % to 25 %) and stearin (~ 15 % to 20 %) as well as a small amount of stearic acid (~ 5 %).

To get a better insight into the adulteration of beeswax with paraffin and/or stearic acid, a PCA was performed including now the prepared calibration samples (described in section 2.3), as demonstrated in **Figure 7**. It can be easily observed that a systematic increase of either paraffin or stearic acid content results in an equivalent spread of the relevant calibration samples away from the tight clustering (pure beeswax) towards pure paraffin and stearic acid reference samples. Hence, it is possible to confirm qualitatively those beeswax samples that are adulterated as well as to perform a rough quantitative estimation of the respective adulteration.

Figure 7b shows an enlarged view of the tight clustering located at the centre of Fig. 7a containing pure beeswax as well as some calibration samples. It can be seen that the calibration samples that are found within the main clustering area contain 0 – 5 % paraffin (CP1 and CP2) and 0 – 1.14 % stearic acid (CA1, CA2 and CA3), demonstrating that the IR spectra of the latter cannot be really differentiated from the ones that are considered to be pure beeswax. It is thus concluded that these concentrations describe the sensitivity levels of IR spectroscopy in the detection of paraffin and stearic acid in beeswax and as such, they can be considered threshold values to determine beeswax adulteration.

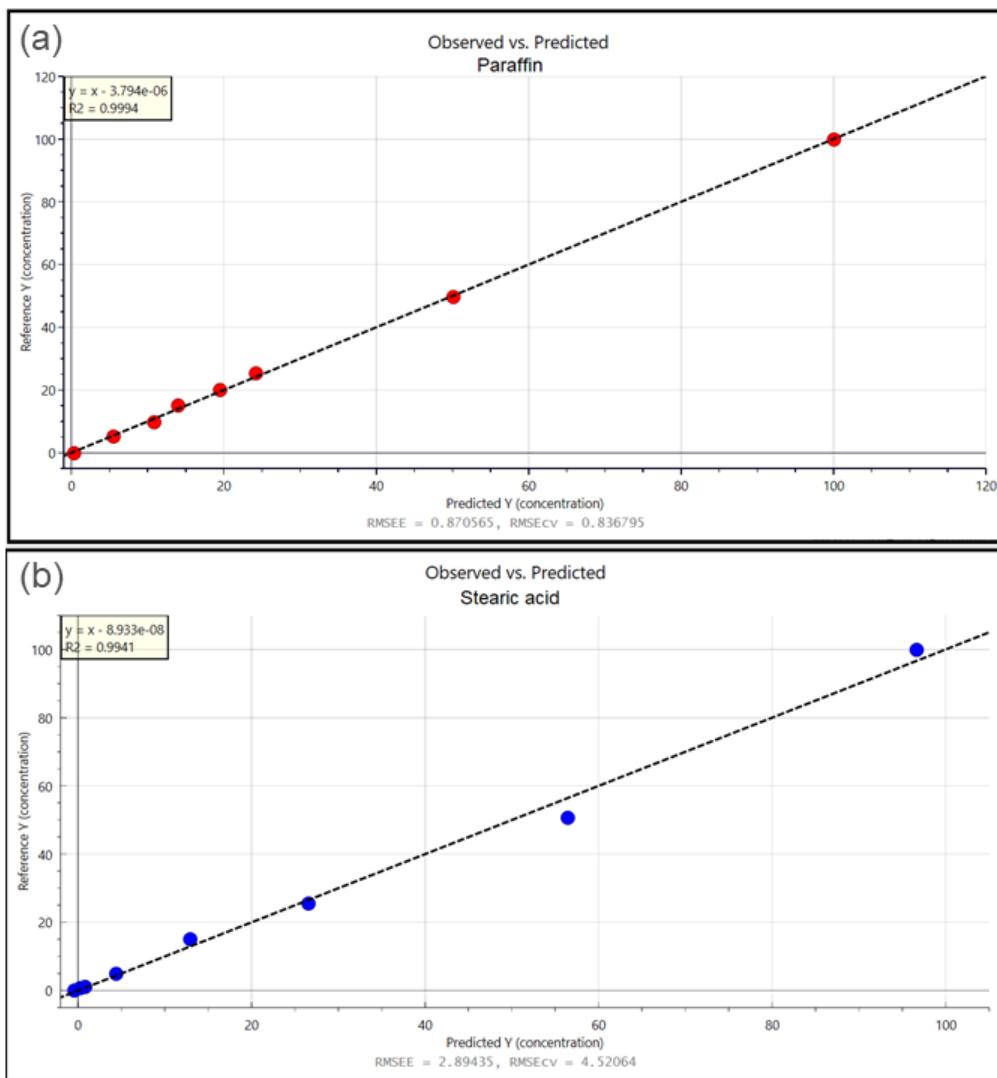
**Figure 7. (a).** PCA scores plot of beeswax, reference and calibration samples. Stearic acid calibration samples are shown in blue, paraffin calibration samples in red and beeswax, tri-stearin and beef tallow in green. **(b).** Enlarged view of the tight clustering area of Figure 7a.



### 3.4 Partial Least Squares (PLS)

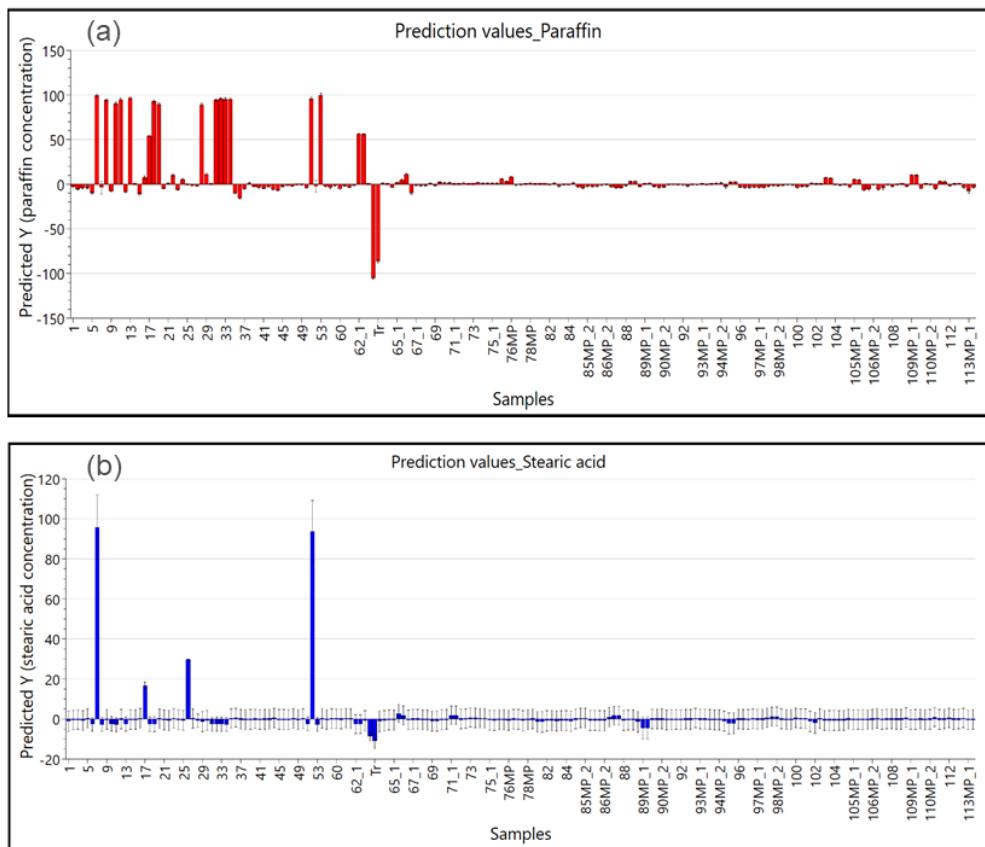
Following the sample screening performed by PCA, a quantification of the concentration of both paraffin and stearic acid is attained by PLS. The first step is to build a model based on the calibration samples that were prepared (described in section 2.3) and assess the quality of these calibrations. Cross validation was performed using the leave-two-out approach in SIMCA software. The relevant calibration curves are shown in **Figure 8**. The calibration samples for both paraffin and stearic acid are well described by a linear fitting with an  $R^2$  value of >0.99, indicating the high quality of the two calibration models. In order to enhance the robustness of the model, it should be developed with sufficient amount of samples that account for most of the variability within the data set.

**Figure 8. (a).** Calibration curve for paraffin. **(b).** Calibration curve for stearic acid.



The second step is to apply these calibration models on the test samples described in Annex 3 in order to predict the concentrations of the two adulterants, as shown **Figure 9**. The upper and lower graphs show the PLS predictions for the concentration of paraffin and stearic acid in beeswax, respectively.

**Figure 9.** (a). Prediction values for paraffin concentrations. (b). Prediction values for stearic acid concentrations.



On one hand, it is observed that several samples seem to contain very high amounts of paraffin (>80 %), while two of them are found to contain ~ 50 %. However, the majority of the investigated samples either contain low amounts of paraffin (<10 %) or no paraffin. It is also interesting to note that the specimens that are characterized as highly adulterated (>80 %) represent commercial samples in the form of pearls and blocks, whereas honeycomb type samples were generally perceived as pure. Few exceptions were solely observed for some honeycomb middle parts with a minor paraffin content.

On the other hand, it is natural to predict a high percentage of stearic acid (>90 %) in samples 7 and 52, since these specimens are pure stearic acid, while only two samples (17 and 26) exhibit moderate amount of stearic acid (10 - 40 %). The rest of the samples showed either low or no traces of stearic acid. Similar to the paraffin case, adulterated specimens 17 and 26 are commercial samples in the form of candles and block. PLS prediction values for adulterated beeswax with paraffin (>5 %) and stearic acid (>1 %) are presented in **Table 3**.

**Table 3.** PLS predictions for paraffin and stearic acid concentrations.

PARAFIN	5-10 %	10-15 %	15-75 %	>75 %
	24,66,76,76MP,103MP ,105MP	29,109MP	17,62	8,10,11,13, 18,19,28,3 1,32,33,34, 51
STEARIC ACID	1-5 %	5-20 %	20-75 %	>75 %
	66,71,87,87MP,98MP	17	26	-

### 3.5 Transferability study

To check the reproducibility of spectral measurements, seven beeswax samples spiked with different concentrations of paraffin and paraffin/stearic acid were prepared and sent to Professor Lidija Svečnjak in the University of Zagreb for IR measurements (Croatian lab). In particular, five samples contained paraffin at concentrations of 2.99, 4.02, 5, 7.09 and 10.03 %, while from the two remaining samples one contained 5.02 % paraffin/4.95 % stearic acid and the other 3.75 % paraffin/2.96 % stearic acid. A standard operating procedure (SOP) document was provided to the Croatian lab to ensure that the spectroscopic measurements were done under the same conditions (see Annex 1).

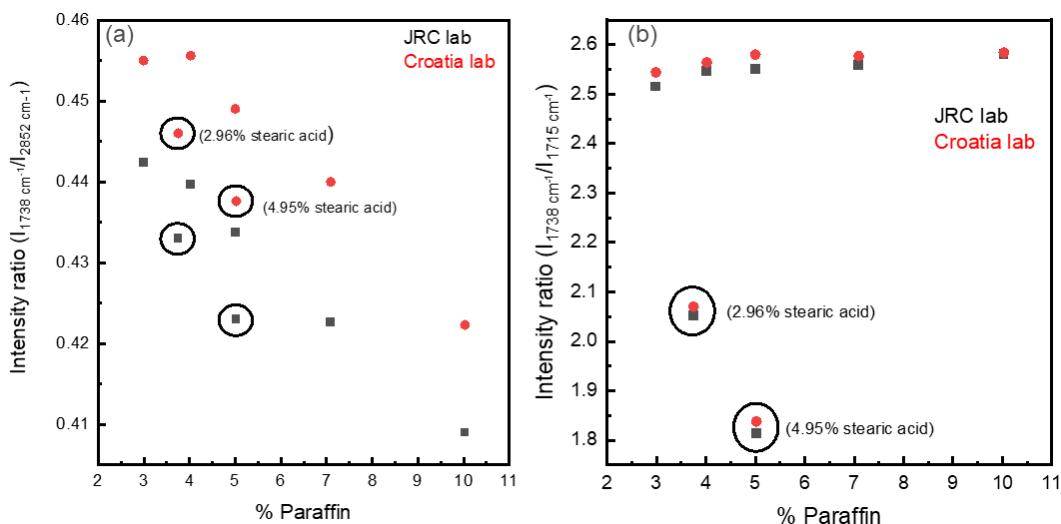
The measurements were performed in triplicates for each of the seven samples and the average values were considered. The data were subsequently processed by calculating the following ratios:

$$1). I_{1738 \text{ cm}^{-1}}/I_{2852 \text{ cm}^{-1}} \text{ and } 2). I_{1738 \text{ cm}^{-1}}/I_{1715 \text{ cm}^{-1}}$$

The IR spectra of beeswax, paraffin and stearic acid show that the evolution of the  $I_{1738 \text{ cm}^{-1}}/I_{2852 \text{ cm}^{-1}}$  intensity ratio is an indicator of the paraffin content, whereas the  $I_{1738 \text{ cm}^{-1}}/I_{1715 \text{ cm}^{-1}}$  ratio is used to determine the stearic acid content. The calculation of the individual peak intensities at 1715, 1738 and 2852  $\text{cm}^{-1}$  was conducted by adding the intensities of two neighbouring wavenumbers below and above these wavenumber values, as previously reported by Maia et al (Miguel Maia et al., 2013).

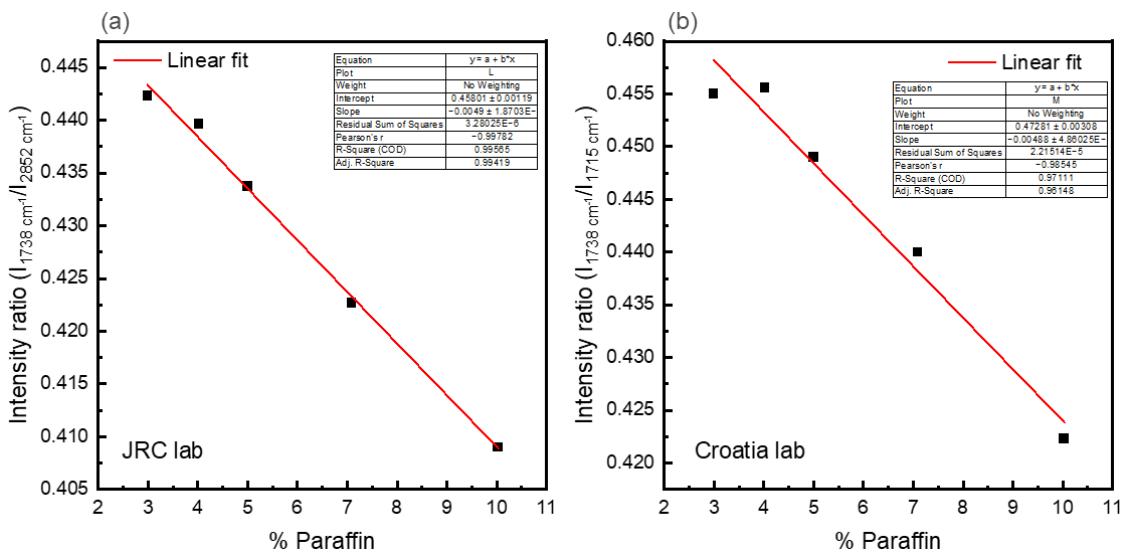
**Figure 10** shows two graphs of both intensity ratios as a function of paraffin content. The two samples that were spiked with stearic acid as well are indicated in the circles. Figure 10a shows that the  $I_{1738 \text{ cm}^{-1}}/I_{2852 \text{ cm}^{-1}}$  ratio values decrease linearly with paraffin content due to decreasing absorption at  $1738 \text{ cm}^{-1}$  attributed to the presence of beeswax. The two samples containing stearic acid deviate from the general linear trend observed for samples spiked solely with paraffin, because stearic acid is also weakly absorbing at  $1738 \text{ cm}^{-1}$ . Figure 10b demonstrates that the  $I_{1738 \text{ cm}^{-1}}/I_{1715 \text{ cm}^{-1}}$  ratio remains almost constant as a function of increasing paraffin content, due to the fact that IR absorption at these two frequencies is exclusively assigned to beeswax with no paraffin interference. However, this ratio is drastically affected in the two stearic acid containing samples, because stearic acid shows a very intense absorption band at  $\sim 1715 \text{ cm}^{-1}$  and hence this is reflected in ratio values even at low stearic acid concentrations. As a result, the latter intensity ratio is a useful indicator to estimate the concentration of stearic acid, while the former intensity ratio is useful to investigate the amount of paraffin.

**Figure 10. (a)**. The evolution of the  $I_{1738 \text{ cm}^{-1}}/I_{2852 \text{ cm}^{-1}}$  ratio as a function of paraffin content. **(b)** The evolution of the  $I_{1738 \text{ cm}^{-1}}/I_{1715 \text{ cm}^{-1}}$  ratio as a function of paraffin content. The data point in the circle represent the two samples that also contain stearic acid.



**Figure 11a** represents the evolution of the  $I_{1738 \text{ cm}^{-1}}/I_{2852 \text{ cm}^{-1}}$  ratio as a function of paraffin content, upon removal of the two samples that also contain stearic acid.

**Figure 11. (a).** The evolution of the  $I_{1738\text{ cm}^{-1}}/I_{2852\text{ cm}^{-1}}$  ratio as a function of paraffin content calculated by the JRC lab. **(b).** The evolution of the  $I_{1738\text{ cm}^{-1}}/I_{2852\text{ cm}^{-1}}$  ratio as a function of paraffin content calculated by the Croatia lab. The samples containing stearic acid are removed.



The  $I_{1738\text{ cm}^{-1}}/I_{2852\text{ cm}^{-1}}$  intensity ratio values exhibit an excellent linearly fitting ( $R^2 > 0.96$ ) for the measurements conducted in both labs and the equations generated were the following:

$$\text{JRC lab: } y = -0.00490 \cdot x + 0.458 \quad \text{Eq. 1}$$

$$\text{Croatia lab: } y = -0.00488 \cdot x + 0.473 \quad \text{Eq. 2}$$

where  $y$  is the intensity ratio value and  $x$  is the % paraffin concentration.

Solving equations 1 and 2 with respect to  $x$  and by replacing  $y$  with the calculated intensity ratio values, we can estimate  $x$  values for both labs. These calculations show that deviations of these  $x$  values between the two labs are less than 12 %, which shows a very good agreement for the estimations of paraffin concentrations among the two labs. Furthermore, the  $I_{1738\text{ cm}^{-1}}/I_{1715\text{ cm}^{-1}}$  values depicted in **Figure 11b** among the two labs are also in excellent agreement. Overall, these findings clearly demonstrate that that transferability was achieved since the method has been applied by an independent laboratory analysing blind samples and correct results were obtained.

## 4 Chromatography based analytical method

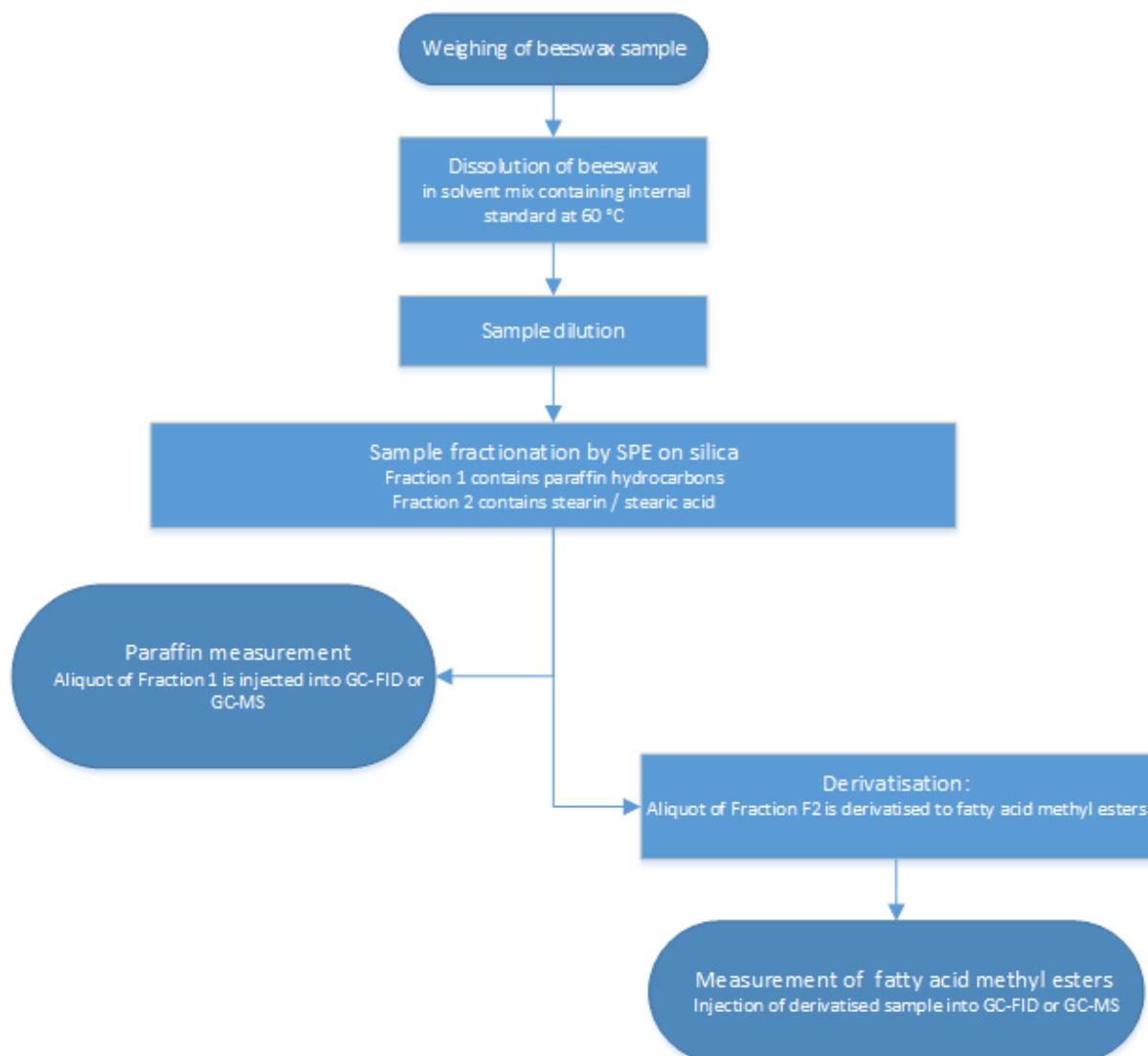
### 4.1 Outline of chromatography based analytical method

The chromatography based analytical methods presented in scientific literature for the determination of paraffin comprise usually the dissolution of the beeswax in an organic solvent, the addition of an internal standard and the measurement of the solution by gas chromatography (Chen et al., 2012; J. J. Jiménez, Bernal, del Nozal, Martín, & Bernal, 2006; M. Maia & Nunes, 2013; Serra Bonvehi & Orantes Bermejo, 2012). Was et al. proposed a sample preparation procedure, which includes fractionation of the sample by solid phase extraction (SPE) on aluminium oxide (Waś et al., 2014a). The latter procedure has the advantage of reducing the load on the chromatography column.

Complete dissolution of the tested beeswaxes was only achieved with solvents respectively solvent mixtures containing at least a certain fraction of halogenated solvents such as dichloromethane.

The main elements of the applied procedure are depicted in **Figure 12**. It offers the measurement of the test samples both by GC-FID and GC-MS. The very details of the analytical procedure are given in Annex 2.

**Figure 12.** Main steps of analytical procedure



## 4.2 Optimizations of sample preparation

### 4.2.1 Solid phase extraction

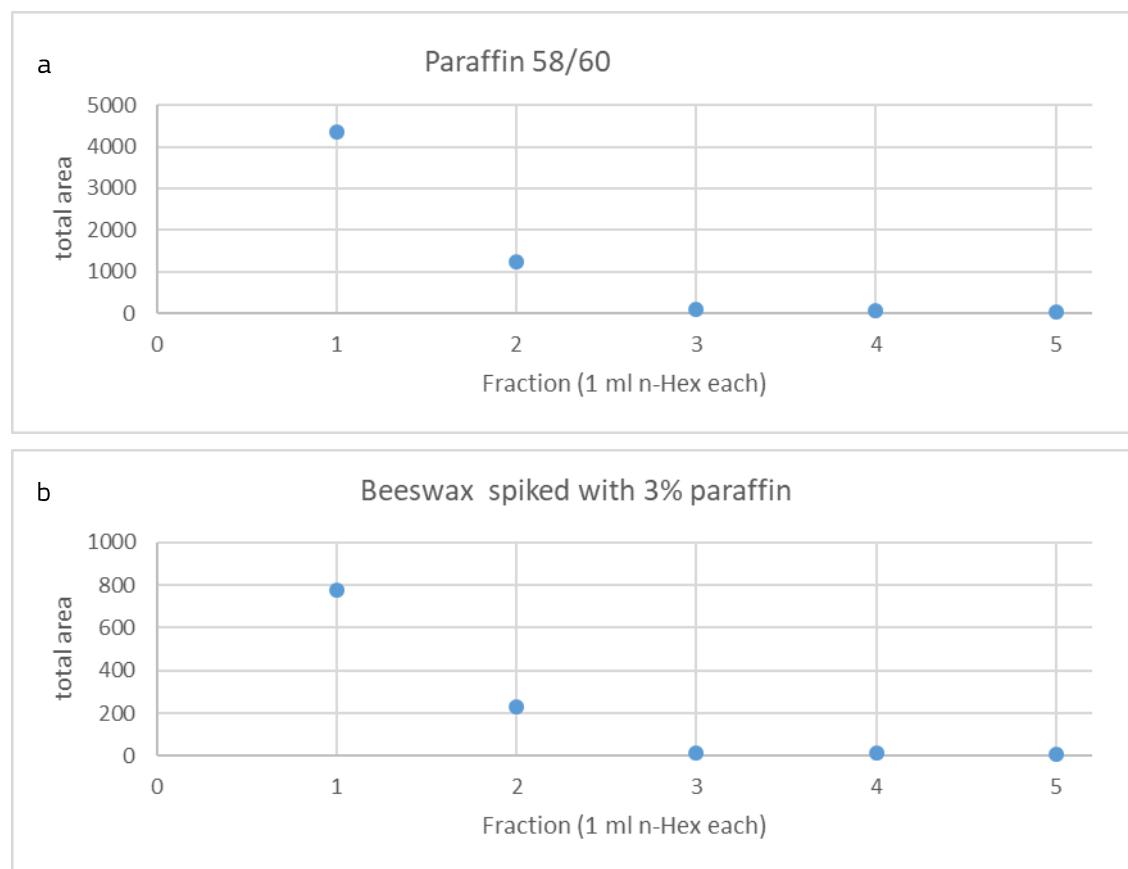
The development of fractionation by solid phase extraction (SPE) was subject to several criteria. It shall separate paraffin *n*-alkanes from the rest of the beeswax matrix, as this separation is beneficial for chromatography column performance if only paraffin contents of beeswax are in the scope of the analyses. Stearin respectively stearic acid shall be contained in a separate fraction.

The fractionation shall be selective and quantitative, in order to achieve reliable analytical results.

Silica is frequently used for the separation of hydrocarbons from other substances as hydrocarbons are only weakly adsorbed.

**Figure 13** displays the cumulative signal intensities of *n*-alkanes with 20 to 40 carbon atoms for a pure paraffin sample (melting point 58 °C to 60 °C) and a beeswax sample spiked with 3 % of paraffin. Both samples were treated according to the standard operating procedure outlined in Figure 12 and presented in detail in Annex 1. The analytical results demonstrated that paraffin *n*-alkanes are eluted from the SPE cartridge within the first two millilitres of *n*-hexane.

**Figure 13.** Elution profile of paraffin *n*-alkanes from 3 ml SPE cartridges containing 500 mg silica;  
a) paraffin 58/60 b) pure beeswax samples spiked with 3 % w/w paraffin



The second fractionation step consisted of the elution of stearin/stearic acid with a solvent mixture of *n*-hexane and ethyl acetate at equal volumes. Experiments showed that stearin/stearic acid is eluted within the first two millilitres of the eluent. An extra volume of 0.5 ml eluent was added to the 2.0 ml of solvent mix in the analysis of beeswax test samples for assuring quantitative elution of the analytes.

#### 4.2.2 Derivatisation of stearin/stearic acid

The direct determination of glycerides from beeswax by gas chromatography may be regarded disproportionate for the purpose of detection of potential adulteration with stearin. For this purpose, it is easier and sufficient to investigate the composition of the ester bound fatty acids than the composition of glycerides itself. This can be achieved by the chemical transformation of the glycerides into fatty acid methyl esters (FAMEs), which is also beneficial regarding chromatographic conditions. A number of transesterification methods are described in literature for the conversion of fatty acids bound in glycerides into FAMEs. The reactions can be selective for bound fatty acids or could cover also free fatty acids. There is also the possibility to discriminate between free and found fatty acids.

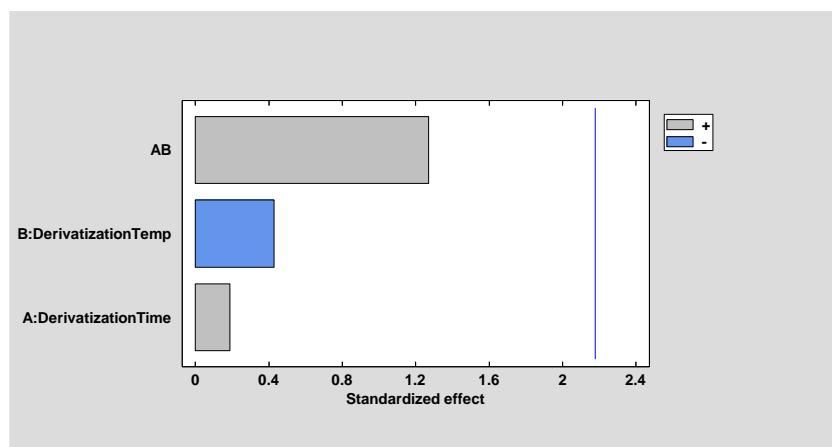
The application of boron trifluoride, a strong Lewis acid, in methanol as reagent for the esterification of free fatty acids to FAMEs respectively transesterification of glycerides into FAMEs allows both the combined and differentiated determination of the contents of free and bound fatty acids. Differentiation between the two groups of substances can be achieved via the reaction time. Free fatty acids react readily within the reagent, whereas the transesterification reaction is much slower. However, discrimination between free and bound fatty acids is not required for the investigation whether a certain beeswax sample is adulterated with stearin/stearic acid.

The purpose of the optimization of the derivatization reaction was to find reaction conditions which allow the combined determination of free and bound fatty acids and which are compatible with routine laboratory practices. Suitable reaction conditions were derived experimentally applying a multilevel factorial experimental design. Studied parameters were derivatization time (10 min to 480 min) and derivatization temperature (40 °C to 90 °C). The reagent was supplied in all experiments in big excess. Solutions of known concentration of both heptadecanoic acid and glyceryl triheptadecanoate were used for the optimization experiments. Stearic acid methyl ester was added as internal standard prior to the measurements of samples by GC-FID. The ratios between heptadecanoic acid methyl ester peak areas and stearic acid methyl ester peak areas were used as response variable in data evaluation.

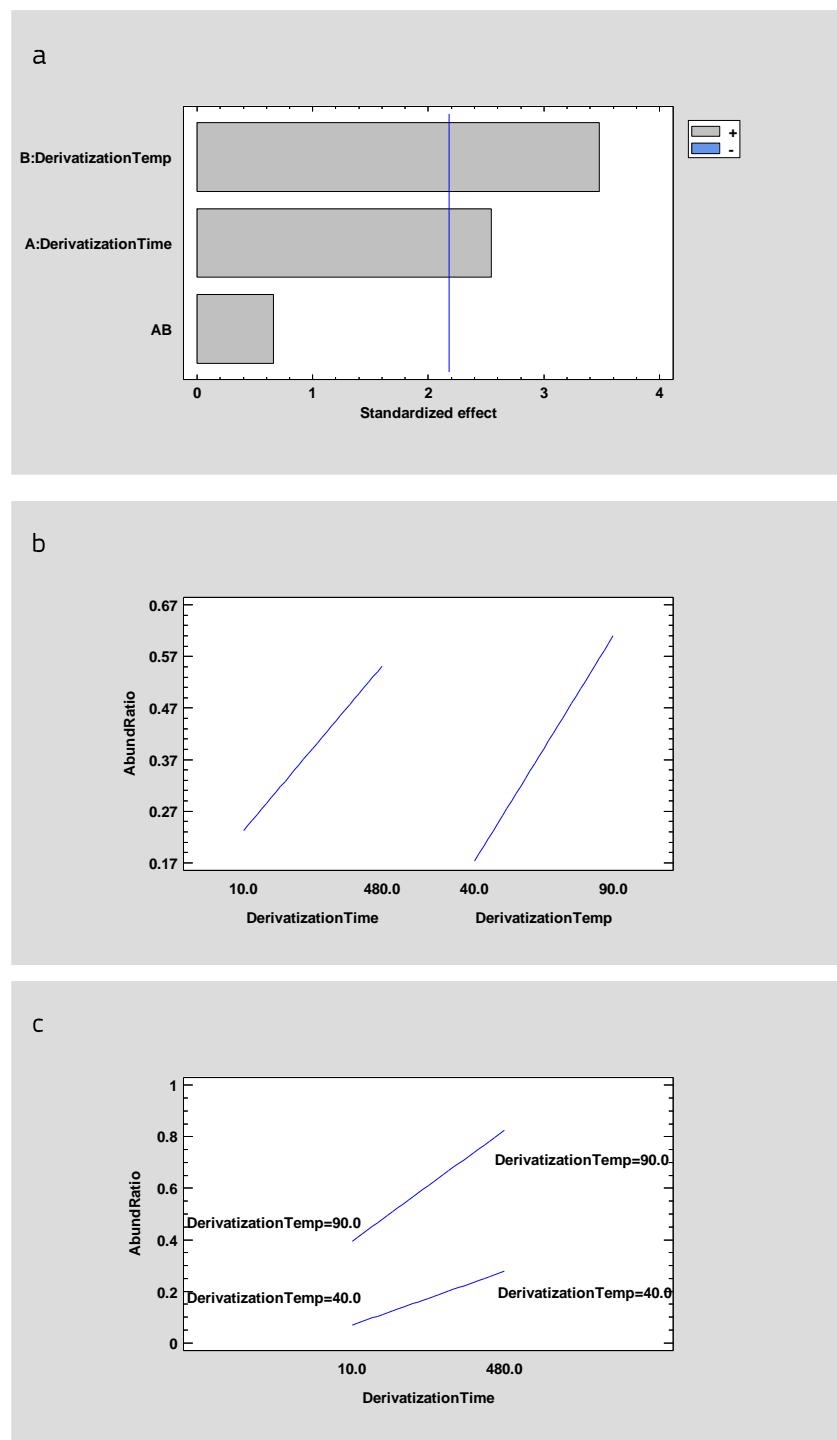
The results of the optimization are depicted in **Figure 14** for the derivatization of heptadecanoic acid and in **Figure 15** for the transesterification of glyceryl triheptadecanoate.

The Pareto chart in Figure 14 demonstrates that neither of the two experimental factors had statistically significant influence (95 % confidence level) on the derivatization of free fatty acids, as none of the bars exceeds the threshold level (indicated by the blue line). The reaction may be considered quantitatively after 10 min at 40 °C. This is different for the transesterification reaction. Both variables had statistical significant effects on the area ratios of heptadecanoic acid methyl ester and stearic acid methyl ester. The reaction yield was highest at the upper parameter levels, as indicated in the main effects and interaction plots.

**Figure 14.** Standardized Pareto chart for the derivatization of heptadecanoic acid



**Figure 15.** Results for the transesterification of glycerol triheptadecanoate  
a: standardized Pareto chart; b: main effects plot; c: interaction plot



Despite a temperature of 90 °C gave best results, the derivatization temperature was lowered for safety reasons to 70 °C for the measurement of beeswax samples. Methanol at 90 °C has a vapour pressure of about 2.5 bars, which would demand the application of special pressure resistant reaction vessels for the transesterification of glycerides. Lowering the temperature to 70 °C halves the vapour pressure, expanding the range of suitable glass ware. In return, the derivatisation time was extended from 8 hours to overnight derivatisation, which is more than 12 hours in normal working hours.

## 4.3 Method validation data

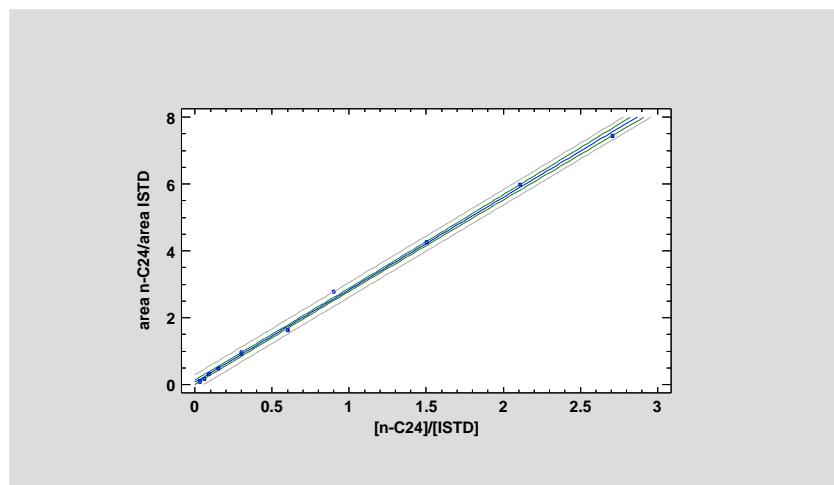
### 4.3.1 Linearity

The linearity of responses were assed from calibration curves. They were created for the paraffinic compounds from C18 to C40 saturated *n*-alkanes in the concentration range of about 0.4 µg/mL to about 36 µg/mL by application of a commercial standard solution containing 1000 µg/mL of each *n*-alkane in *n*-hexane. The standard stock solution was prepared gravimetrically by differential weighing of the contents of two ampoules of the commercial standard solution and combining them in a 50 ml volumetric flask. This stock solution was used to create ten calibration solutions. All calibration solutions were analysed in duplicate. Calibration curves were determined by linear regression. The working range of the method was defined based on the calibration curves. Calibration data were utilized to investigate linearity. A lack-of-fit test did not reveal any statistical significant lack of fit.

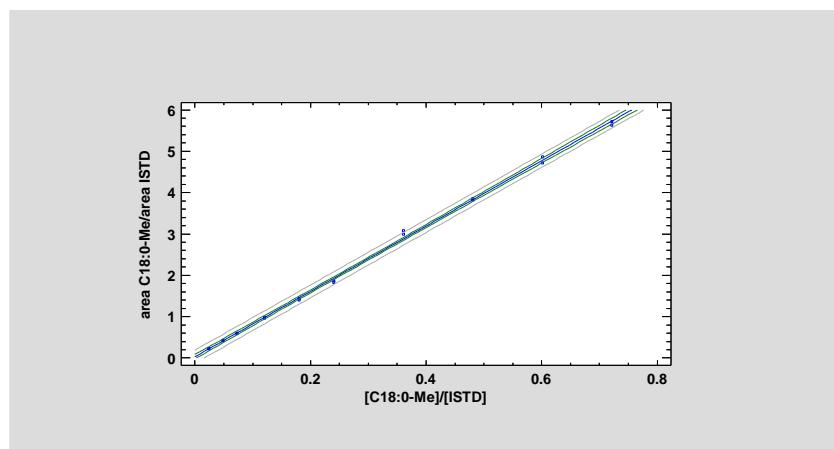
A second set of calibration curves were created for the subsequent quantification of stearin/stearic acid using standard solutions of palmitic acid methyl ester (C16:0-Me), stearic acid methyl ester (C18:0-Me), and oleic acid methyl ester (C18:1-Me) at a concentration range of about 1.0 µg/mL to about 30.0 µg/ml.

All calibration curves were linear within the concentration range given in the standard operating procedure (SOP). Graphs of the calibration functions for *n*-tetracosane (C<sub>24</sub>) and stearic acid methyl ester (C18:0-Me) are given in **Figure 16** and **Figure 17** as an example.

**Figure 16.** Calibration curve for *n*-tetracosane



**Figure 17.** Calibration curve for stearic acid methyl ester



### **4.3.2 LOD and LOQ**

A beeswax sample contaminated with low levels of paraffin and providing low signal intensities for stearic acid methyl ester, the derivative of stearin, was used in the experiments for the estimation of these two method performance parameters. The measurement results for stearic acid methyl ester were converted and expressed as contents of stearic acid. Ten replicates were analysed in total and the standard deviation of the ratio of analyte signal to internal standard signal was used for the calculations according to the equations shown above. LODs were calculated for individual *n*-alkanes as well as for stearic acid derived from tristearin.

Based on the experimental design, limit of detection (LOD) and limit of quantification (LOQ) were calculated as:

$$\text{LOD} = \frac{2.8 \times SD}{b} \quad \text{and} \quad \text{LOQ} = \frac{10 \times SD}{b}$$

SD: residual standard deviation

b: slope of calibration curve

The LODs for the individual even numbered *n*-alkanes were generally below 2.5 mg/g and the LOD for the sum of even numbered *n*-alkanes in the range of C14 to C40 (*n*-alkanes below C14 were not present in beeswax samples) was 19 mg/g.

The LOD for stearic acid was 0.1 mg/g and the LOQ 0.4 mg/g.

### **4.3.3 Matrix effect**

In order to investigate the presence of matrix effects matrix matched calibration curves were compared to calibration curves of analytes in pure solvent. Matrix extract solutions were obtained from a native beeswax sample, by combining the fractions of six replicate analyses of this sample. Two replicates were prepared for each matrix matched calibration solution, which were then analysed by GC-FID.

Significant differences between the matrix free and matrix matched calibration curves were not found.

### **4.3.4 Selectivity**

Selectivity is a method performance parameter that provides information on the influence of sample components on the measurement of the respective analyte.

Procedural blank samples comprising DCM:*n*-hexane (1:5) both with and without internal standard squalane were analysed by GC-FID in two replicates.

A fully operational system did not provide interfering peaks. However, additional peaks were detected in the chromatograms in case of an aged or damaged chromatographic column. They eluted along the chromatographic run in regular time intervals with the same peak pattern. Replacing the damaged column remediated this problem. The mentioned interferences affect especially analyses by GC-FID. Attention has to be given in the determination of fatty acid methyl esters by GC-FID to potential co-elution of analytes with other beeswax constituents.

The determination of stearin/stearic acid by GC-MS is less affected by interferences due to the increased selectivity of the measurements.

### **4.3.5 Repeatability and Intermediate Precision**

Repeatability expresses the precision under fixed conditions in which the analysis is performed by the same operator, same instrument, same method within a short period of time, whereas intermediate precision includes a higher degree of variability. For the estimation of the repeatability standard deviation, subsamples of a particular low contaminated beeswax sample were analysed in three replicates under repeatability conditions.

This process was repeated for the estimation of intermediate precision on another three days spanning a period of four weeks.

The results were evaluated for homogeneity of variances by Cochran test. Repeatability and intermediate precision estimates were calculated by one way ANOVA and expressed as relative standard deviation.

**Table 4** provides the estimates of repeatability relative standard deviations and intermediate precision relative standard deviations for the homologous series of *n*-eicosane (C20) to *n*-triacontane (C30), as well as for palmitic acid (C16:0) and stearic acid (C18:0), both determined as their methyl esters.

**Table 4.** Repeatability and intermediate precision relative standard deviations for the homologous series of *n*-alkanes from *n*-eicosane to *n*-triacontane and palmitic and stearic acid

	RSD <sub>r</sub>	RSD <sub>I</sub>
	%	%
C20	5.5	11.1
C21	3.7	17.0
C22	5.0	13.0
C23	2.6	10.0
C24	2.2	11.8
C25	9.7	9.9
C26	1.9	8.6
C27	1.8	6.2
C28	2.1	8.7
C29	1.7	8.0
C30	1.7	8.0
C16:0	4.4	9.5
C18:0	9.3	12.6

The obtained repeatability relative standard deviations (RSD<sub>r</sub>) were for the majority of compounds in the low percentage range, while the intermediate precision relative standard deviations (RSD<sub>I</sub>) were in the range 6 % - 17 %.

Repeatability and intermediate precision of the analysis of palmitic acid methyl ester and stearic acid methyl ester, the latter is the derivative of stearin/stearic acid, were calculated by means of ANOVA from replicate measurements of a beeswax test sample on different days. The repeatability relative standard deviations were for palmitic acid methyl ester 4.4 % and for stearic acid methyl ester 9.3 %, whereas intermediate precision relative standard deviations were 9.3 % and 12.6 % respectively.

The obtained relative standard deviations indicate satisfactory method performance.

## 4.4 Typical contents and thresholds

### 4.4.1 Paraffin *n*-alkanes

The composition of beeswax is as for many other natural products subject to a certain level of variability.

Jiménez et al. and Waś et al. presented data on the composition of natural beeswax (Juan José Jiménez et al., 2007; Waś et al., 2014a). Jiménez et al. obtained the data from the analysis of in total 22 white or yellow coloured beeswax samples, whereas Waś et al analysed wax from 7 light coloured combs by GC-MS. In the same year, Waś et al published a larger study on the hydrocarbon composition of *Apis mellifera* wax (Waś et al., 2014b). This study comprised 47 light coloured beeswax samples and 23 dark coloured (2-3 years old) beeswax samples (**Table 6**), collected in two different years and within one particular year at two different times. Small, but statistically significant differences in the contents of some *n*-alkanes were found for light and dark bees wax samples and for samples from two different years, but not for samples from the same sampling year.

The two groups expressed the *n*-alkane contents as grams per 100 grams of sample (percentage of mass). Jiménez et al. proposed to use this data as guidance for the assessment of the authenticity of beeswax. The data published by Jiménez et al. and the data obtained Waś et al. by GC-MS is presented in **Table 5** complemented with data generated by the JRC from 32 honey comb sidewall samples.

**Table 5.** Concentration guidance values and results of JRC

Jiménez et al. 2007				Waś et al. 2014					JRC 2023					
	min	max		min	max	mean	Stdev	RSD		min	max	mean	Stdev	RSD
	%m	%m		%m	%m	%m	%m	%		%m	%m	%m	%m	%
C20	0.03	0.13	C20	0.02	0.05	0.03	0.01	29.8	C20		0.25	0.06	0.06	99.3
C21	0.19	0.61	C21	0.04	0.11	0.07	0.02	33.6	C21		0.04	0.02	0.01	65.4
C22	0.02	0.13	C22	0.03	0.09	0.05	0.02	46.9	C22		0.08	0.03	0.03	97.1
C23	0.69	1.33	C23	0.23	0.43	0.29	0.07	23.8	C23	0.14	0.34	0.21	0.05	22.3
C24	0.04	0.18	C24	0.04	0.12	0.08	0.04	44.5	C24		0.15	0.04	0.03	87.7
C25	1.26	1.78	C25	0.75	1.12	0.95	0.18	18.5	C25	0.34	0.87	0.58	0.11	19.3
C26	0.18	0.35	C26	0.10	0.22	0.15	0.04	29.3	C26	0.07	0.26	0.11	0.04	36.0
C27	2.55	3.2	C27	2.98	3.47	3.27	0.18	5.3	C27	2.01	3.18	2.63	0.38	14.6
C28	0.14	0.37	C28	0.08	0.18	0.12	0.03	28.5	C28	0.02	0.17	0.07	0.03	38.3
C29	1.87	2.68	C29	1.99	2.35	2.14	0.13	6.2	C29	1.15	1.87	1.53	0.19	12.6
C30	0.11	0.31	C30	0.08	0.2	0.12	0.04	31.8	C30	0.01	0.12	0.06	0.03	43.1
C31	1.62	2.45	C31	1.66	2.28	1.94	0.21	10.8	C31	0.96	1.80	1.30	0.18	14.2
C32	0.04	0.14	C32	0.04	0.12	0.07	0.03	44.0	C32	0.00	0.11	0.02	0.03	155.1
C33:1	1.19	1.82							C33:1*	1.74	2.68	2.31	0.24	10.6
C33	0.34	0.72	C33	0.39	0.65	0.50	0.08	16.8	C33	0.16	0.35	0.25	0.05	21.3
C34			C34		0.03				C34		0.11	0.03	0.03	109.1
C35	0.01	0.09	C35		0.03				C35		0.09	0.01	0.03	212.6
Total	10.28	16.29	Total	9.08	10.86	9.81	0.66	6.7	Total	7.33	13.06	9.40	1.15	12.2

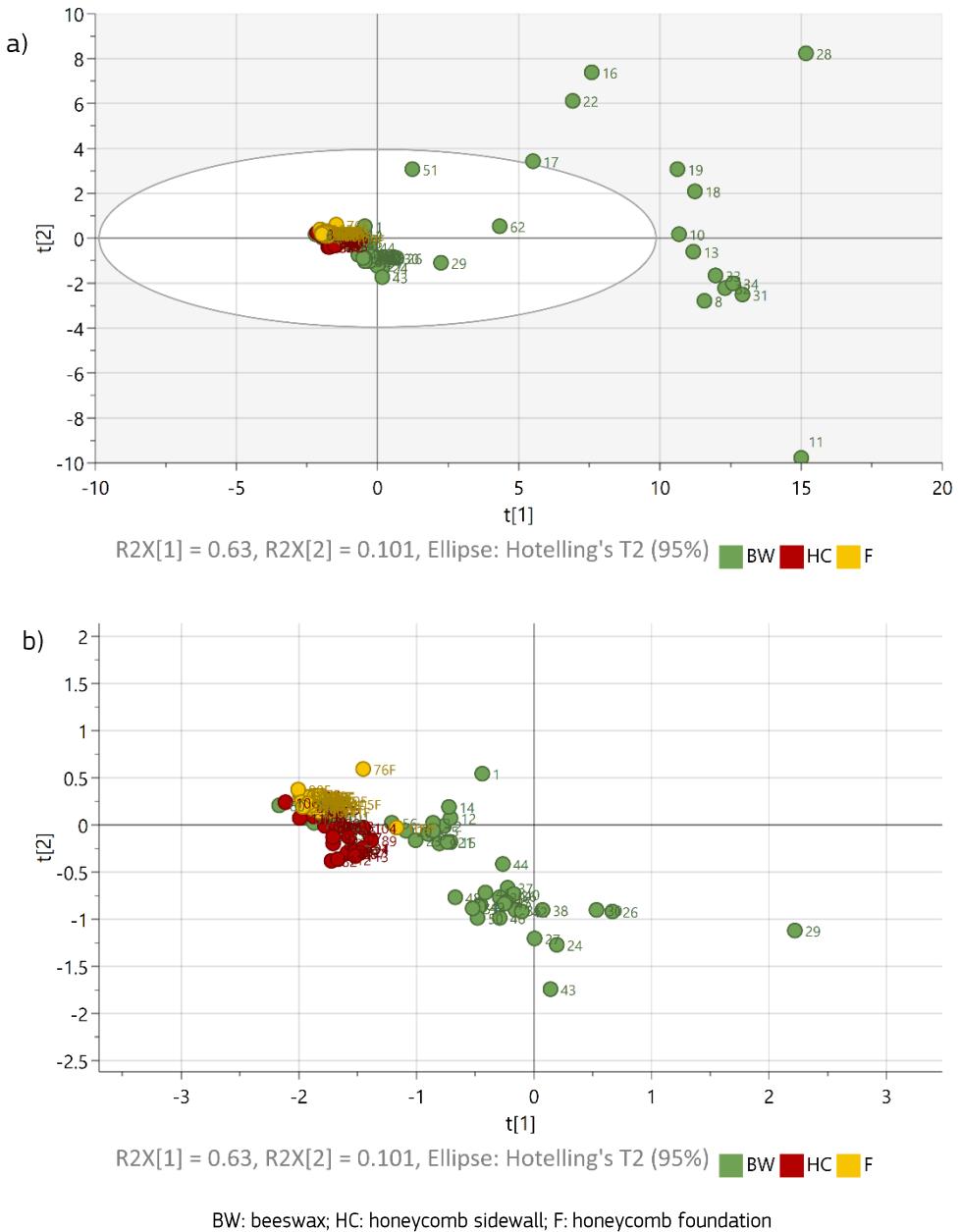
\* quantified with response factors for C33

**Table 6.** *n*-Alkane contents determined in light and dark beeswax samples

	Waś et al. 2014									
	light wax					dark wax				
	min	max	mean	Stdev	RSD	min	max	mean	Stdev	RSD
	%m	%m	%m	%m	%	%m	%m	%m	%m	%
C20	0.02	0.05	0.03	0.01	32.5	0.01	0.06	0.03	0.01	53.7
C21	0.03	0.08	0.05	0.02	30.8	0.03	0.1	0.05	0.02	48.1
C22	0.02	0.06	0.04	0.01	31.6	0.02	0.09	0.04	0.02	52.2
C23	0.12	0.56	0.3	0.11	35.2	0.32	0.68	0.45*	0.12	26.2
C24	0.03	0.12	0.07	0.02	33.9	0.04	0.13	0.07	0.03	43.3
C25	0.42	1.47	0.82	0.19	22.7	0.78	1.37	1.03*	0.18	18.0
C26	0.06	0.18	0.12	0.02	19.1	0.09	0.22	0.13	0.05	34.4
C27	2.44	4.4	3.23	0.43	13.2	2.78	4.17	3.03	0.44	12.6
C28	0.06	0.14	0.10	0.02	20.2	0.08	0.19	0.12*	0.04	29.6
C29	1.68	2.73	2.23	0.22	10.1	1.96	2.59	2.3	0.14	6.0
C30	0.05	0.11	0.08	0.01	17.8	0.06	0.19	0.12*	0.04	31.9
C31	1.53	2.64	1.93	0.24	12.6	1.69	2.25	1.88	0.12	6.3
C32	0.01	0.07	0.03	0.01	40.6	0.02	0.12	0.07*	0.03	44.1
C33	0.31	0.67	0.45	0.1	21.5	0.24	0.76	0.38	0.15	38.7
C34		0.03				0.03				
C35		0.03				0.03				
Total	8.27	11.66	9.5	0.72	7.6	9.46	10.84	10.22	0.36	3.5

The *n*-alkane contents measured in the different studies overlap to some extent. However the results demonstrate that deducing threshold values for authenticity assessment is not trivial. **Figure 18a** displays the score plot of the principle component analysis (PCA) of the measurements of individual *n*-alkanes ranging from *n*-hexadecane (C16) to *n*-tetracontane (C40) in 136 samples of commercial beeswax (green circles), honeycomb sidewalls (red circles), and honeycomb foundations (yellow circles). The honeycomb sidewall samples and honeycomb foundation samples fall together as shown in **Figure 18b**, which is a magnification of Figure 18a. The honeycomb sidewall and honeycomb foundation samples are clearly separated from beeswax samples containing elevated amounts of paraffin *n*-alkanes. Several of these samples were outside the 95% confidence ellipse (Figure 18a).

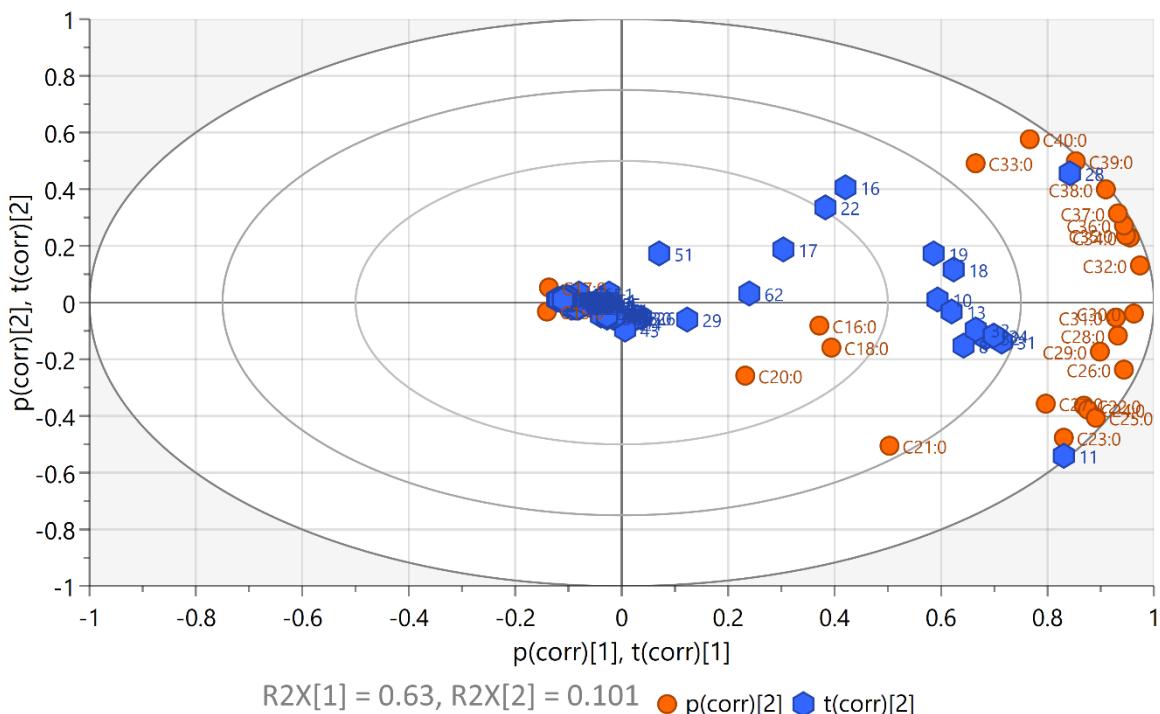
**Figure 18.** a) PCA score plot of individual *n*-alkanes measured in 136 samples; b) magnification of score plot of Figure 18a



The measurement of 24 *n*-alkanes provides the possibility to discriminate native beeswax from beeswax containing elevated levels of *n*-alkanes. However, their entire application is a burden to the analyst and requires the evaluation of the measurements via multivariate statistical models, which is not the most preferred option. Additionally, among these *n*-alkanes are substances with little discrimination power respectively substances which have equal discrimination power to other *n*-alkanes. Hence, the aim was to minimize the number of

substances to be measured without compromising discrimination power. **Figure 19** presents the biplot (a combination of scores and loadings plots) of the measurements of *n*-alkanes in 136 samples. The scores are the same as in Figure 18. They are overlaid with the normalized loadings of the individual *n*-alkanes. The position of the scores of samples (blue hexagons) with elevated *n*-alkane content is influenced by variables lying in the same direction in the loadings plot (orange circles). This means that the *n*-alkanes listed at the right boarder are most suitable to identify elevated *n*-alkane levels in beeswax. The majority of the measured *n*-alkanes are close to the outer ellipse. Among them are the even numbered *n*-alkanes from *n*-docosane (C22) to *n*-dotriacontane (C32), which may be expected at low content levels in native beeswax samples as well as longer *n*-alkanes, which normally do not occur in beeswax (*n*-pentatriacontane (C35) and higher). Hence the sole presence of the latter is an indication of the presence of paraffin, whereas the contents of the earlier, natural beeswax *n*-alkanes are only topped up by the addition of paraffin.

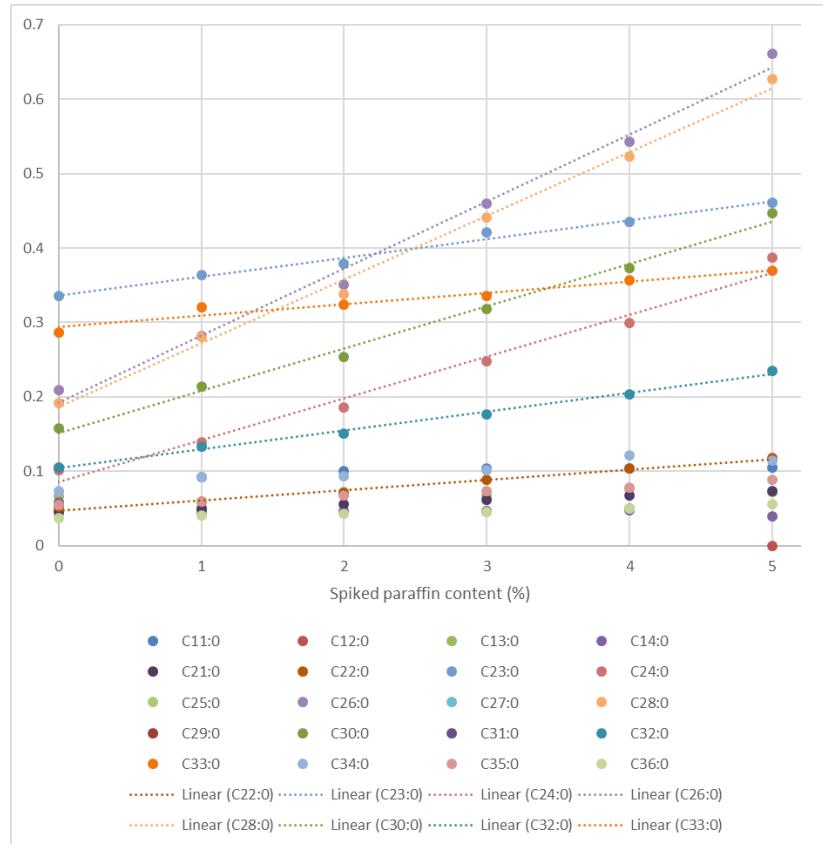
**Figure 19.** Biplot of PCA of individual *n*-alkanes measured in 136 samples



Another aspect for the selection of marker substances is the sensitivity of the essay. Sensitivity is expressed in chemical analysis by the change of signal intensity in function of the analyte concentration, and is equal to the slope of a calibration curve. For identifying beeswax *n*-alkanes with the highest change of signal intensity, a native beeswax sample was spiked with a commercial paraffin (with melting point similar to that of beeswax). The spiking levels were one to five percent of mass. The measurement results were plotted for each substance against the spiking level and subjected to linear regression. **Figure 20** compiles the results for a number of *n*-alkanes. The largest slopes were found for the *n*-alkanes *n*-tetracosane (C24), *n*-hexacosane (C26), *n*-octacosane (C28) and *n*-triacontane (C30). Hence these substances are good candidates for the assessment of the authenticity of beeswax. The graphs for these four substances demonstrate that the difference in signal intensities between the native beeswax sample and the same beeswax sample spiked with up to 5 % of paraffin is sufficiently large to meet the requirement of identifying the addition of minimum 5% of paraffin.

The typical contents of these four *n*-alkanes in native beeswax were assessed based on 78 samples, comprising beeswax samples, honeycomb side walls, and honeycomb foundations. These samples were identified from the conducted PCA. They are grouped together in Figure 18b. Descriptive statistics parameters of the results of this assessment are presented in Table 7 for different combinations of *n*-alkanes together with total *n*-alkane contents, and contents of even numbered *n*-alkanes and odd numbered *n*-alkanes.

**Figure 20.** Linear regression of *n*-alkanes measured in beeswax spiked with different levels of paraffin



**Table 7** presents descriptive statistics for the contents of different groups of *n*-alkanes of 78 beeswax samples. The samples comprised commercial products and beeswax obtained directly from honeycombs.

**Table 7:** Descriptive statistics for *n*-alkane contents of beeswax samples

		Sum of all alkanes	sum odd	sum even	C24+C26+C28+C30	C24+C26+C28	C26+C28	C26
Mean	mg/g	108.1	92.3	8.6	5.0	3.9	3.0	1.7
Stdev	mg/g	27.5	29.0	5.2	3.6	2.7	2.2	1.1
Mean + 2x Stdev	mg/g	163.1	150.3	18.9	12.1	9.3	7.4	4.0
90 percentile	mg/g	148.7	133.4	16.4	11.1	8.7	6.6	3.4
95 percentile	mg/g	151.2	134.3	19.0	11.8	10.0	7.3	3.9
99 percentile	mg/g	155.9	135.0	20.7	13.4	10.4	8.0	4.5
Max	mg/g	157.4	135.2	22.5	13.4	10.5	8.2	4.6

Jiménez et al proposed to use the content ratios between adjacent even and odd numbered *n*-alkanes as criterion for authenticity assessment in place of pure contents(Juan José Jiménez et al., 2007). This proposal was studied and the effect of content ratios for the discrimination of native and adulterated beeswax was evaluated. Different *n*-alkane ratios respectively the ratios of the sum of different *n*-alkanes were tested. The ratio of the sum of contents of *n*-tetracosane, *n*-hexacosane, *n*-octacosane and *n*-triacontane and of the sum of contents of *n*-tricosane, *n*-pentacosane, *n*-heptacosane and *n*-nonacosane provided the highest number of samples exceeding the threshold given by the mean value of 78 samples considered authentic plus two times their standard deviation. Respective data are plotted in **Figure 21**.

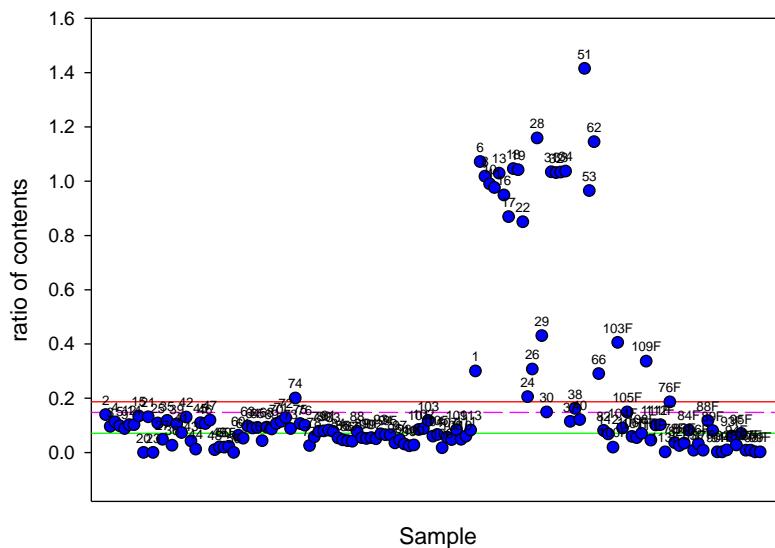
Thirty three samples exceeded the threshold given by the mean ratio plus two times the standard deviation of the ratios. This is comparable to the result obtained if the ratios of the sum of contents of the four even numbered *n*-alkanes and the four odd numbered *n*-alkanes are replaced by the sum of contents of even numbered *n*-alkanes in the range of *n*-hexadecane to *n*-tetracontane. The plot for the sum of contents of even numbered *n*-alkanes is shown in **Figure 22**. The plot of content ratios (Figure 21) demonstrates that some low contaminated samples are clearer separated from the native samples compared to the plot in Figure 22.

The ratio of contents of even numbered and odd numbered n alkanes can be used only for the identification of adulterations, whereas the sum of contents of even numbered n-alkanes allows also to conclude on the level of adulteration, as it may be assumed that contents of even numbered alkanes and odd numbered alkanes are in paraffin at equal content levels. Based on this assumption, the dotted line in Figure 21 represents a 5 % contamination level. The threshold value for this level was derived by Equation 3, which considers the variability of n-alkane contents in native beeswax:

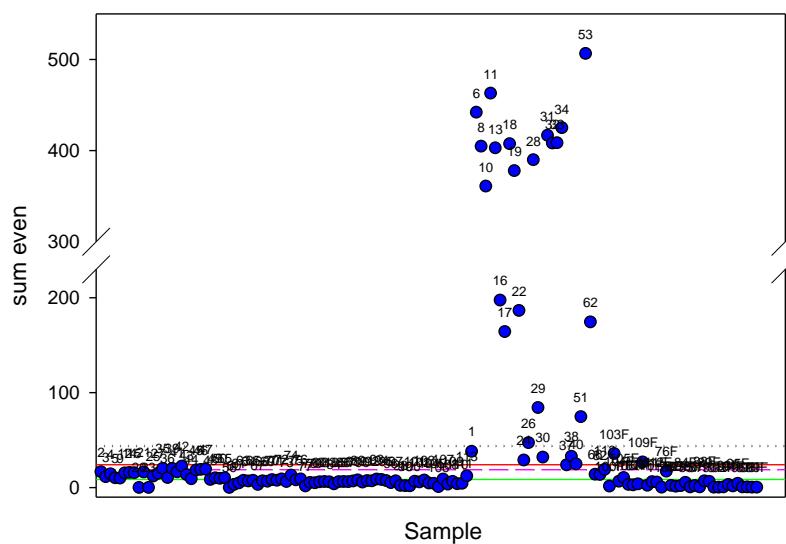
$$T = 0.95 * (\bar{x} + 2 * \sigma_x) + 5 * a \quad \text{Eq. 3}$$

where  $T$  is the threshold value (mg/g),  $\bar{x}$  is the mean value of the sum of contents of even numbered n-alkanes contained in native beeswax (mg/g),  $\sigma_x$  is the standard deviation of the sum of contents of even numbered n-alkanes contained in native beeswax (mg/g),  $a$  is the adulteration level in percent

**Figure 21.** Plot of the ratio of the sum of contents of (C24 + C26 + C28 + C30) and of the sum of contents of (C23 + C25 + C27 + C29)



**Figure 22.** Plot of the sum of contents (mg/g) of even numbered  $n$ -alkanes from hexadecane to tetracontane.



Absolute clarity about the level of adulteration with paraffin can be obtained only when all *n*-alkanes in the range between *n*-hexadecane and *n*-tetracosane are quantified.

Threshold values for total *n*-alkane contents which consider the statistical variability of contents may be derived for different adulteration levels by Equation 4, which is analogous to Equation 3.

$$T_y = 0.95 * (\bar{y} + 2 * \sigma_y) + 10 * a \quad \text{Eq. 4}$$

where  $T_y$  is the threshold value for total *n*-alkane content (mg/g),  $\bar{y}$  is the mean value of the total contents of *n*-alkanes contained in native beeswax (mg/g),  $\sigma_y$  is the standard deviation of total *n*-alkane contents in native beeswax (mg/g),  $a$  is the adulteration level in percent

A value for total *n*-alkane content of 205 mg/g is proposed as threshold for the identification of adulterations with paraffin exceeding the 5% level with a high level of probability.

#### 4.4.2 Stearin/stearic acid

Stearin is in the narrow sense the triacylglyceride of three units of stearic acid. However, the term is also used for the solid fraction of an oil or fat which melts at higher temperature. This is for example the case for palm stearin, which is composed of triglycerides rich in palmitic acid and stearic acid. Stearin is not volatile and difficult to analyse. The preferred analytical approach is to convert the fatty acids bound in glycerides into fatty acid methyl esters and measure the fatty acids methyl esters by gas chromatography with flame ionisation or mass spectrometric detection. The amount of corresponding free fatty acid is calculated via the ratio of molecular masses of the free fatty acid and of the fatty acid methyl ester.

Stearic acid is also converted to stearic acid methyl ester prior to measurement by gas chromatography.

Native beeswax contains both free fatty acids as well as fatty acids bound both in glycerides and to long chain linear alcohols. The latter form wax esters, the major components of beeswax.

Different authors reported on the contents of palmitic acid and stearic acid in beeswax. Jiménez et al. proposed total palmitic acid contents in the range of 150 to 192 mg/g and total stearic acid contents in the range of 4.3 mg/g to 8.7 mg/g beeswax as guidance values for the assessment of beeswax authenticity (Juan José Jiménez et al., 2007).

Results of own measurements on the test samples used in this study provided much broader ranges of palmitic acid and stearic acid contents. They are reasoned by the intentional addition of stearin or stearic acid to the samples, resulting in high stearic acid contents, or by the dilution of beeswax with e.g. paraffin.

The plot of stearic acid contents against palmitic acid contents of all samples, which is depicted in **Figure 23** revealed a clear separation of samples with low from samples with high stearic acid contents. Most of the stearic acid contents were low, whereas the palmitic acid contents were distributed along the abscissa. Samples with very low palmitic acid contents were adulterated with paraffin, which dilutes natural beeswax constituents.

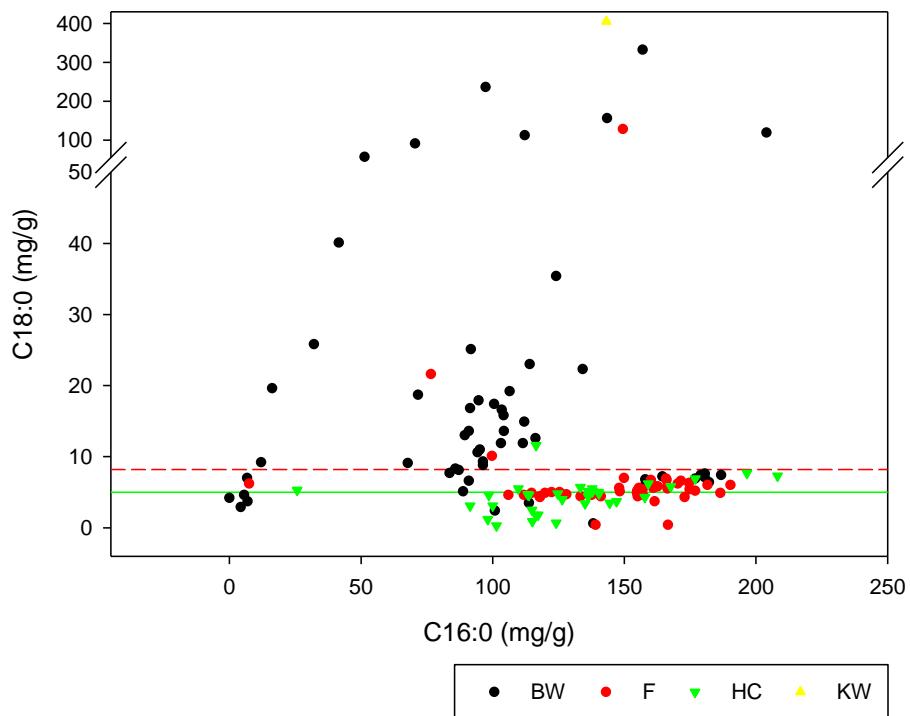
For the estimation of realistic native stearic acid contents, descriptive statistics was performed on the results for seventy five samples, which were considered as authentic. These samples represented mainly honeycomb sidewall samples, foundation plate samples, and some commercial beeswax samples. They all had palmitic acid contents in the range between 100 mg/g and 200 mg/g and stearic acid contents below 10 mg/g (see Figure 23). The green line in Figure 23 indicates the mean value of stearic acid contents of 75 samples considered as authentic, whereas the red dotted lines represents this value plus two times the standard deviation (from Table 7).

The result of descriptive statistics are presented in **Table 8**. The mean and maximum contents of palmitic acid and of stearic acid of the investigated samples agree well with the contents reported by Jiménez et al. (Juan José Jiménez et al., 2007).

**Table 8:** Descriptive statistics of palmitic acid and stearic acid contents in 75 authentic beeswax samples

	C16:0 mg/g	C18:0 mg/g
Min	85.8	0.3
Mean	143.8	5.0
Median	145.8	5.1
Stdev	29.1	1.6
Max	208.2	8.3
90 Percentile	180.1	7.0
95 Percentile	183.7	7.3
99 Percentile	199.7	7.9
Mean + 2x Stdev	202.1	8.3

**Figure 23:** Plot of stearic acid contents against palmitic acid contents



BW: beeswax, F: honeycomb foundation, HC: honeycomb, KW: commercial synthetic wax

The addition of stearic acid to native beeswax increases the measured stearic acid directly by the amount of added stearic acid. Therefore a measured stearic acid content of more than 58.3 mg/g allows to conclude with a high level of probability on an adulteration level of minimum 5%.

About 96 % of the mass of stearin (tristearin) stems from stearic acid. The resulting threshold value for 5% adulteration is 56.3 mg/g, which is almost equal to the value given in the paragraph before. Therefore, for reasons of simplicity it is suggested to apply the same threshold value both for tristearin and stearic acid.

#### **4.5 Method transferability study**

Two laboratories, one from Italy and one from Portugal, expressed their interest in participating in the study on the transferability of the gas chromatographic analysis method. The Portuguese laboratory resigned from the study still before test samples and consumables were dispatched. A replacement could not be found on short notice. The Italian laboratory was supplied in June 2023 with a set of test samples and the majority of chemicals and consumables, which are required for the implementation of the analytical method. This laboratory could not yet perform the analyses. They reasoned it with problems with the mass spectrometer. However, they promised to provide analysis results in the coming weeks.

The study demonstrated that it becomes more and more difficult to find laboratories, which participate in collaborative studies on a voluntary basis.

## 5 Conclusions

The present report compiles the outcome of investigation of beeswax adulteration by paraffin and/or stearic acid/stearin by spectroscopic and chromatographic methods. In particular, ATR-IR spectroscopy combined with multivariate statistics was mainly used as a screening tool to evaluate the presence of paraffin and/or stearic acid/stearin in beeswax and to provide estimations on their respective concentrations. Complete statistical evaluation of the ATR data was performed using both PCA and PLS methods. PCA was successfully applied for the identification of clusters of authentic and suspicious samples as well as for performing a preliminary estimation of the corresponding adulteration levels, when calibration samples were included in the analysis. This estimation is in line with the PLS results where the final quantification of the paraffin and stearic acid concentrations are performed.

The PLS predictions demonstrated that commercial beeswax samples are regularly more prone to adulteration than unprocessed honeycombs and that for the latter only a minor number of the middle parts exhibited moderate levels of adulteration. The developed multivariate statistical approach is in agreement with previous works reporting detection limits of ~ 5 % for paraffin and ~ 1 % for stearic acid in beeswax. These findings constitute a clear demonstration that ATR-IR is fast and very reliable screening method to investigate beeswax adulteration. The successful transfer of the method to an independent laboratory demonstrated the ruggedness of the protocol, which is important for application in official controls.

The chromatographic methods allow both the identification, confirmation well as the estimation of the levels of adulteration of beeswax with paraffin and with stearin/stearic acid. Both flame ionisation and mass spectrometric detection are possible. However, in order to make use of rather uniform response factors and mass spectrometric selectivity, it is suggested to apply gas chromatography with flame ionisation detection for the measurement of paraffin n-alkanes and gas chromatography mass spectrometry for the determination of stearic acid methyl ester, which is the measurand derived from stearin/stearic acid. Method performance parameters demonstrate the ability to detect and quantify low levels of adulterations. The performance of the developed method exceeds the criterion of detectability of adulteration levels of minimum 5 % by weight. However, threshold values were established for the 5 % target level from the analysis results of more than 70 beeswax samples, which were considered authentic. Considering the analytical variability and the natural variability of the contents of native beeswax of both *n*-alkane and stearic acid, the derived threshold values are 205 mg/g for the sum of paraffin *n*-alkane contents of *n*-alkanes in the range between *n*-hexadecane and *n*-tetracontane and about 58 mg/g for stearic acid. Above these threshold values, contamination of beeswax exceeding 5 % by weight may be assumed with high probability.

In a next step, the chromatographic analysis method should be validated by collaborative trial in order to make it fully applicable for official controls. The standardisation of the method by an international standardisation organisation might be envisaged subsequently.

## References

- Castro, A. V., Medici, S. K., Sarlo, E. G., & Eguaras, M. J. (2010). Agregado de parafina en ceras estampadas y su efecto sobre el labrado de panales y viabilidad de las crías de Apis Mellifera. *Zootecnia Tropical*, 28, 353-361.
- Chen, F., Zheng, C., Chen, L., Zhao, J., Xue, X., & Wu, L. (2012). Gas Chromatographic-Mass Spectrometry and Chemometric Analysis for Detection and Quantification of Paraffin in Beeswax. *Food Science and Technology Research*, 18(1), 17-24. doi:10.3136/fstr.18.17
- Council of Europe. (2020). European Pharmacopoeia 10th ed. Supplement 2. In (10th Edition ed.). Strasbourg, France: EDQM - European Directorate for the Quality of Medicine & Healthcare.
- El Agrebi, N., Svecnjak, L., Horvatinec, J., Renault, V., Rortais, A., Cravedi, J.-P., & Saegerman, C. (2021). Adulteration of beeswax: A first nationwide survey from Belgium. *PLoS ONE*, 16(9), e0252806. doi:10.1371/journal.pone.0252806
- European Commission. (2011). *Commission Regulation (EU) No 142/2011 of 25 February 2011 implementing Regulation (EC) No 1069/2009 of the European Parliament and of the Council laying down health rules as regards animal by-products and derived products not intended for human consumption and implementing Council Directive 97/78/EC as regards certain samples and items exempt from veterinary checks at the border under that Directive*. Official Journal of the European Union. Vol. L 54, pp. 1-254.
- European Food Safety Authority. (2020). Risk assessment of beeswax adulterated with paraffin and/or stearin/stearic acid when used in apiculture and as food (honeycomb). *EFSA Supporting Publications*, 17(5), 1859E. doi:10.2903/sp.efsa.2020.EN-1859
- European Union. (2002). *Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety*. Official Journal of the European Union. Vol. L 31, pp. 1-50.
- European Union. (2008). *Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives*. Official Journal of the European Union. Vol. L 354, pp. 16.
- European Union. (2009). *Regulation (EC) No 1069/2009 of the European Parliament and of the Council of 21 October 2009 laying down health rules as regards animal by-products and derived products not intended for human consumption and repealing Regulation (EC) No 1774/2002 (Animal by-products Regulation)*. Official Journal of the European Union. Vol. L 300, pp. 1.
- Freund, M. I., & Mózes, G. (1982). *Paraffin products properties, technologies, applications*. Amsterdam, The Netherlands : Elsevier Scientific Pub. Co.
- Hepburn, H. R., Pirk, C. W. W., & Duangphakdee, O. (2014). The Chemistry of Beeswax. *Honeybee Nests*. doi:10.1007/978-3-642-54328-9\_16
- Idris Nor, A., & Mat Sahri, M. (2007). Utilization of palm oil and palm products in shortenings and margarines: Palm oil. *European Journal of Lipid Science and Technology*, 109(4), 422-432.
- Jiménez, J. J., Bernal, J. L., del Nozal, M. a. J., Martín, M. a. T., & Bernal, J. (2006). Sample preparation methods for beeswax characterization by gas chromatography with flame ionization detection. *Journal of Chromatography A*, 1129(2), 262-272. doi:10.1016/j.chroma.2006.06.098
- Jiménez, J. J., Bernal, J. L., del Nozal, M. J., Martín, M. T., & Toribio, L. (2009). Identification of adulterants added to beeswax: Estimation of detectable minimum percentages. *European Journal of Lipid Science and Technology*, 111(9), 902-911. doi:10.1002/ejlt.200800263
- Jiménez, J. J., Bernal, J. L., del Nozal, M. J., Toribio, L., & Bernal, J. (2007). Detection of beeswax adulterations using concentration guide-values. *European Journal of Lipid Science and Technology*, 109(7), 682-690. doi:10.1002/ejlt.200600308
- Jones, R. N., McKay, A. F., & Sinclair, R. G. (1952). Band Progressions in the Infrared Spectra of Fatty Acids and Related Compounds1. *Journal of the American Chemical Society*, 74(10), 2575-2578. doi:10.1021/ja01130a034

- Kitagawa, I., Sugai, M., & Kummerow, F. A. (1962). Infrared spectra and gas chromatography of some oxygenated fatty acid derivatives. *Journal of the American Oil Chemists Society*, 39(4), 217-222. doi:10.1007/BF02635824
- Maia, M., Barros, A. I. R. N. A., & Nunes, F. M. (2013). A novel, direct, reagent-free method for the detection of beeswax adulteration by single-reflection attenuated total reflectance mid-infrared spectroscopy. *Talanta*, 107, 74-80. doi:10.1016/j.talanta.2012.09.052
- Maia, M., & Nunes, F. M. (2013). Authentication of beeswax (*Apis mellifera*) by high-temperature gas chromatography and chemometric analysis. *Food Chemistry*, 136(2), 961-968. doi:10.1016/j.foodchem.2012.09.003
- Muscat, D., Tobin, M. J., Guo, Q., & Adhikari, B. (2014). Understanding the distribution of natural wax in starch-wax films using synchrotron-based FTIR (S-FTIR). *Carbohydrate Polymers*, 102, 125-135. doi:10.1016/j.carbpol.2013.11.004
- Reybroeck, W. (2017, 30 June 2017). *Field trial: effect of the addition of a mixture of stearic acid and palmitic acid (called stearin) to beeswax on the development of the worker bee brood*. ILVO, Melle, Belgium.
- Serra Bonvehi, J., & Orantes Bermejo, F. J. (2012). Detection of adulterated commercial Spanish beeswax. *Food Chemistry*, 132(1), 642-648. doi:10.1016/j.foodchem.2011.10.104
- Socrates, G. (2004). *Infrared and Raman Characteristic Group Frequencies: Tables and Charts, 3rd Edition*: Wiley.
- Špaldoňová, A., Havelcová, M., Lapčák, L., Machovič, V., & Titěra, D. (2021). Analysis of beeswax adulteration with paraffin using GC/MS, FTIR-ATR and Raman spectroscopy. *Journal of Apicultural Research*, 60(1), 73-83. doi:10.1080/00218839.2020.1774152
- Svečnjak, L., Baranović, G., Vinceković, M., Prđun, S., Bubalo, D., & Gajger, I. T. (2015). An Approach for Routine Analytical Detection of Beeswax Adulteration Using FTIR-ATR Spectroscopy. *Journal of Apicultural Science*, 59(2), 37-49. doi:10.1515/jas-2015-0018
- Svečnjak, L., Chesson, L. A., Gallina, A., Maia, M., Martinello, M., Mutinelli, F., . . . Waters, T. A. (2019). Standard methods for *Apis mellifera* beeswax research. *Journal of Apicultural Research*, 58(2), 1-108. doi:10.1080/00218839.2019.1571556
- Svečnjak, L., Nunes, F. M., Matas, R. G., Cravedi, J.-P., Christodoulidou, A., Rortais, A., & Saegerman, C. (2021). Validation of analytical methods for the detection of beeswax adulteration with a focus on paraffin. *Food Control*, 120, 107503. doi:10.1016/j.foodcont.2020.107503
- Tanner, N., & Lichtenberg-Kraag, B. (2019). Identification and Quantification of Single and Multi-Adulteration of Beeswax by FTIR-ATR Spectroscopy. *European Journal of Lipid Science and Technology*, 121(12). doi:10.1002/ejlt.201900245
- Tinto, W. F., Elufioye, T. O., & Roach, J. (2017). Chapter 22 - Waxes. In S. Badal & R. Delgoda (Eds.), *Pharmacognosy* (pp. 443-455). Boston: Academic Press.
- Tulloch, A. P. (1980). Beeswax—Composition and Analysis. *Bee World*, 61(2), 47-62. doi:10.1080/0005772X.1980.11097776
- Waś, E., Szczesna, T., & Rybak-Chmielewska, H. (2014a). Determination of Beeswax Hydrocarbons by Gas Chromatography with a Mass Detector (GC -MS ) Technique. *Journal of Apicultural Science*, 58. doi:10.2478/jas-2014-0015
- Waś, E., Szczesna, T., & Rybak-Chmielewska, H. (2014b). Hydrocarbon Composition of Beeswax (*Apis Mellifera*) Collected from Light and Dark Coloured Combs. *Journal of Apicultural Science*, 58(2), 99-106. doi:10.2478/jas-2014-0026
- Waś, E., Szczesna, T., & Rybak-Chmielewska, H. (2016). Efficiency of GC-MS method in detection of beeswax adulterated with paraffin. *Journal of Apicultural Science*, 60(1), 145-162. doi:10.1515/jas-2016-0012

## List of abbreviations and definitions

ATR	attenuated total reflectance
BF	beef fat
BW	beeswax or product made of beeswax
C14	<i>n</i> -tetradecane
C16	<i>n</i> -hexadecane
C20	<i>n</i> -eicosane
C21	<i>n</i> -heneicosane
C22	<i>n</i> -docsane
C23	<i>n</i> -tricosane
C24	<i>n</i> -tetracosane
C25	<i>n</i> -pentacosane
C26	<i>n</i> -hexacosane
C27	<i>n</i> -heptacosane
C28	<i>n</i> -octacosane
C29	<i>n</i> -nonacosane
C30	<i>n</i> -triacontane
C31	<i>n</i> -hentriacontane
C32	<i>n</i> -dotriacontane
C33	<i>n</i> -tritriacontane
C33:1	<i>n</i> -tritriacontene
C34	<i>n</i> -tetratriacontane
C35	<i>n</i> -pentatriacontane
C40	<i>n</i> -tetracontane
C16:0	palmitic acid
C16:0-Me	palmitic acid methyl ester
C18:0	stearic acid
C18:0-Me	stearic acid methyl ester
DCM	dichloromethane
F	honeycomb foundation
FAME	fatty acid methyl ester
FID	flame ionisation detection
FTIR	Fourier transform infrared spectroscopy
GC	gas chromatograph
HC	honeycomb sidewall
MS	mass spectrometer
PCA	principle component analysis
PLS	partial least squares
SNV	standard normal variate

SPE	solid phase extraction
Stdev	standard deviation
Sum odd	sum of contents of <i>n</i> -alkanes in the range from hexadecane to tetracontane with odd number of carbon atoms in the chain
Sum even	sum of contents of <i>n</i> -alkanes in the range from hexadecane to tetracontane with even number of carbon atoms in the chain
Tr	tristearin

## List of figures

<b>Figure 1.</b> Schematic representation of a single bounce ATR experiment on beeswax.....	7
<b>Figure 2.</b> IR spectra of paraffin, beeswax, stearic acid, beef fat samples and tri-stearin. Spectra (except paraffin) are shifted for clarity.....	8
<b>Figure 3.</b> Contribution of various principal components in explained variance.....	9
<b>Figure 4.</b> PCA scores plot of commercial and reference samples. Reference stearic acid samples (samples 7 and 52) are shown in the blue circle, reference paraffin (samples 6 and 53) and highly paraffin adulterated beeswax samples are shown in the red circle, pure beeswax samples are included in the purple circle and tri-stearin (Tr)/beef fat (BF) reference samples are included in the black circle.....	9
<b>Figure 5. (a).</b> PCA scores plot (t3 vs t1) of commercial and reference samples. <b>(b).</b> PCA scores plot (t3 vs t2) of commercial and reference samples.....	10
<b>Figure 6.</b> IR spectra of beeswax, beef fat, tri-stearin and samples 16, 22. Spectra are shifted for clarity.....	11
<b>Figure 7. (a).</b> PCA scores plot of beeswax, reference and calibration samples. Stearic acid calibration samples are shown in blue, paraffin calibration samples in red and beeswax, tri-stearin and beef tallow in green. <b>(b).</b> Enlarged view of the tight clustering area of figure 8a.....	12
<b>Figure 8. (a).</b> Calibration curve for paraffin. <b>(b).</b> Calibration curve for stearic acid.....	13
<b>Figure 9. (a).</b> Prediction values for paraffin concentrations. <b>(b).</b> Prediction values for stearic acid concentrations.....	14
<b>Figure 10. (a).</b> The evolution of the $I_{1738\text{ cm}^{-1}}/I_{2852\text{ cm}^{-1}}$ ratio as a function of paraffin content. <b>(b)</b> The evolution of the $I_{1738\text{ cm}^{-1}}/I_{1715\text{ cm}^{-1}}$ ratio as a function of paraffin content. The data point in the circle represent the two samples that also contain stearic acid.....	15
<b>Figure 11. (a).</b> The evolution of the $I_{1738\text{ cm}^{-1}}/I_{2852\text{ cm}^{-1}}$ ratio as a function of paraffin content calculated by the JRC lab. <b>(b).</b> The evolution of the $I_{1738\text{ cm}^{-1}}/I_{2852\text{ cm}^{-1}}$ ratio as a function of paraffin content calculated by the Croatia lab. The samples containing stearic acid are removed.....	16
<b>Figure 12.</b> Main steps of analytical procedure.....	17
<b>Figure 13.</b> Elution profile of paraffin <i>n</i> -alkanes from 3 ml SPE cartridges containing 500 mg silica; a) paraffin 58/60 b) pure beeswax samples spiked with 3 % w/w paraffin.....	18
<b>Figure 14.</b> Standardized Pareto chart for the derivatization of heptadecanoic acid.....	19
<b>Figure 15.</b> Results for the transesterification of glyceryl triheptadecanoate a: standardized Pareto chart; b: main effects plot; c: interaction plot.....	20
<b>Figure 16.</b> Calibration curve for <i>n</i> -tetracosane.....	21
<b>Figure 17.</b> Calibration curve for stearic acid methyl ester.....	21
<b>Figure 18.</b> a) PCA score plot of individual <i>n</i> -alkanes measured in 136 samples; b) magnification of score plot of Figure 8a.....	25
<b>Figure 19.</b> Biplot of PCA of individual <i>n</i> -alkanes measured in 136 samples.....	26
<b>Figure 20.</b> Linear regression of <i>n</i> -alkanes measured in beeswax spiked with different levels of paraffin.....	27
<b>Figure 21.</b> Plot of the ratio of the sum of contents of (C24 + C26 + C28 + C30) and of the sum of contents of (C23 + C25 + C27 + C29).....	28
<b>Figure 22.</b> Plot of the sum of contents (mg/g) of even numbered <i>n</i> -alkanes from hexadecane to tetracosane.....	28
<b>Figure 23:</b> Plot of stearic acid contents against palmitic acid contents.....	30

## List of tables

<b>Table 1.</b> Calibration samples for paraffin and stearic acid with nominal concentrations.....	6
<b>Table 2.</b> Vibrational assignments of the main bands present in the IR spectra.....	8
<b>Table 3.</b> PLS predictions for paraffin and stearic acid concentrations.....	14
<b>Table 4.</b> Repeatability and intermediate precision relative standard deviations for the homologous series of <i>n</i> -alkanes from <i>n</i> -eicosane to <i>n</i> -triacontane and palmitic and stearic acid.....	23
<b>Table 5.</b> Concentration guidance values and results of JRC.....	24
<b>Table 6.</b> <i>n</i> -Alkane contents determined in light and dark beeswax samples.....	24
<b>Table 7:</b> Descriptive statistics for <i>n</i> -alkane contents of beeswax samples.....	27
<b>Table 8:</b> Descriptive statistics of palmitic acid and stearic acid contents in 75 authentic beeswax samples...	30

## **Annexes**

### **Annex 1. Standard operating procedure for the determination of paraffin and stearic acid in beeswax by Attenuated Total Reflectance Infrared Spectroscopy (ATR-IR).**

#### **Standard Operating Procedure for the Detection of Paraffin and Stearic Acid in beeswax by Attenuated Total Reflectance Infrared Spectroscopy (ATR-IR).**

##### **1. Scope**

This document describes the standard operating procedure (SOP) to be used to determine paraffin and/or stearic acid concentration in beeswax using ATR-IR spectroscopy.

##### **2. Principle**

Beeswax samples spiked with paraffin and/or stearic acid are prepared for spectroscopic investigation. Each sample is melted on a hot plate and is subsequently transferred on an ATR heated stage to be measured. The detection of paraffin and stearic acid is performed by calculating relative intensity ratios of specific peaks in the spectrum used as markers.

##### **3. Reagents and standards**

Use only reagents of recognised analytical quality/grade, unless otherwise specified.

###### **3.1. Isopropanol (cleaning of the ATR crystal), CAS number: 67-63-0**

##### **4. Safety**

Protective equipment such as laboratory coat and safety glasses have to be used. Handling of isopropanol shall be performed under the fume hood using suitable gloves. Persons using these instructions shall be familiar with normal laboratory practice. It is the responsibility of the user of these instructions to apply safety and health practices that are in agreement with the local requirements.

##### **5. Apparatus**

5.1. Heating plate that can reach 85°C.

5.2. ATR-IR spectroscopy apparatus with a diamond ATR crystal equipped with a heating stage.

5.3. Computer coupled to the apparatus for instrument control and data evaluation.

5.4. Conditions for the ATR measurements: 4 cm<sup>-1</sup>resolution, 24 scans, 4000 – 400 cm<sup>-1</sup> spectral range.

## **6. Preparation of the test sample**

### **6.1. Test sample**

The test sample consists of solid beeswax samples spiked with different concentrations of paraffin and stearic acid provided into small glass vials. Each glass vial must be subsequently placed on a heated plate at 85°C for 1 hour to achieve complete and homogeneous melting.

### **6.2. Test portion**

One droplet of test sample is transferred on the diamond ATR crystal to perform a single spectroscopic measurement as described in 7.2.

## **7. Procedure**

### **7.1. Preliminary test**

Prior to the measurements, a preliminary check needs to be done following the next steps:

- 7.1.1. Clean the ATR diamond crystal with isopropanol (3.1) to remove any potential impurities that may have remained from a previous experiment.
- 7.1.2. Conduct a performance qualification (PQ) test to ensure that the instrument is properly set for measurements.
- 7.1.3. Set the temperature of the heating stage at 85°C that is needed to perform the measurements in liquid state.

### **7.2. Spectroscopic determination**

The spectroscopic measurements are performed following the next steps:

- 7.2.1. A background measurement with no sample on the crystal is taken as reference.
- 7.2.2. A droplet of the tested sample (test portion) is poured on the diamond crystal covering its entire surface and a sample measurement is taken. Three replicate measurements are considered for each test sample and each replicate measurement is performed on a single droplet. The spectrum is recorded in absorbance units.
- 7.2.3. The droplet is removed and the crystal is thoroughly cleaned with isopropanol to prepare for the next measurement. Proper cleaning of the crystal is checked before the next measurement by performing a blank test (for details see section 7.3).

Each test sample must be analysed in triplicate. Each replicate measurement must be performed on an independent droplet following following steps 7.2.1 to 7.2.3.

### 7.3. Verification of crystal cleanliness after sample measurement

It is important to verify the cleanliness of the diamond crystal before the next sample measurement by performing a blank test. After cleaning the diamond crystal, a “sample measurement” is conducted with no physical sample in place and the corresponding absorbance spectrum is generated using the last background spectrum. The resulting absorbance spectrum should be zero across the entire wavenumber range.

## 8. Data analysis

### 8.1. Calculation of intensity ratios

The intensity ratios of the peaks:

$$1). I_{1738 \text{ cm}^{-1}} / I_{2851 \text{ cm}^{-1}}$$

and

$$2). I_{1738 \text{ cm}^{-1}} / I_{1715 \text{ cm}^{-1}}$$

are calculated for every measurement. For the calculation of the individual peak intensities at 1715, 1738 and 2851  $\text{cm}^{-1}$ , the intensities of two neighbouring wavenumbers below and above these wavenumber values are also considered in the following way [2]:

$$I_{1715 \text{ cm}^{-1}} = (I_{1711 \text{ cm}^{-1}} + I_{1713 \text{ cm}^{-1}} + I_{1715 \text{ cm}^{-1}} + I_{1717 \text{ cm}^{-1}} + I_{1719 \text{ cm}^{-1}}) / 5$$

$$I_{1738 \text{ cm}^{-1}} = (I_{1734 \text{ cm}^{-1}} + I_{1736 \text{ cm}^{-1}} + I_{1738 \text{ cm}^{-1}} + I_{1740 \text{ cm}^{-1}} + I_{1742 \text{ cm}^{-1}}) / 5$$

and

$$I_{2851 \text{ cm}^{-1}} = (I_{2847 \text{ cm}^{-1}} + I_{2849 \text{ cm}^{-1}} + I_{2851 \text{ cm}^{-1}} + I_{2853 \text{ cm}^{-1}} + I_{2855 \text{ cm}^{-1}}) / 5$$

### 8.2. Reporting of results

The values of the calculated intensity ratios are reported in a table for each replicate measurement (R1,R2,R3) and each test sample.

## 9. References

1. "[List of Classifications, Agents classified by the IARC Monographs, Volumes 1–124](#)". IARC Monographs on the Evaluation of Risk to Humans. IARC. July 7, 2019. Retrieved July 14, 2019.
2. Maia. M , Barros Ana I.R.N.A. and Nunes F.M. A novel, direct, reagent-free method for the detection of beeswax adulteration by single-reflection attenuated total reflectance mid-infrared spectroscopy. *Talanta*, 2013, (107) 74-80.

**Annex 2. Standard operating procedure for the determination of paraffin and stearin/stearic acid in beeswax by gas chromatography**

**Determination of paraffin and stearin/stearic acid in beeswax by gas chromatography**

**1 Scope**

This standard operation procedure is used for the determination of paraffin n-alkanes from octadecan (C18) to tetracontane (C40) and of the fatty acids methyl esters (FAME) palmitic acid methyl ester (C16:0-Me), oleic acid methyl ester (C18:1-Me), and of stearic acid methyl ester (C18:0-Me) in beeswax and beeswax products. The measurement of each of the paraffin n-alkanes is performed by gas chromatography with either flame ionisation detection (GC-FID) or mass spectrometric detection. The fatty acid methyl esters are measured by either gas chromatography with flame ionisation detection (GC-FID) or gas chromatography mass spectrometry (GC-MS).

**2 Method principle**

A small portion of beeswax is dissolved in a solvent mix, which is further diluted. A portion of the diluted samples is fractionated by solid phase extraction. The fraction containing paraffin n-alkanes is measured by gas chromatography with flame ionisation or mass spectrometric detection. The second fraction is further derivatised to yield fatty acid methyl esters which are measured either gas chromatography with flame ionisation detection or by gas chromatography mass spectrometry. Internal standardisation is used for the quantification of the analytes.

**3 Safety**

Applicable laboratory safety procedures as well as personal protection equipment (lab coat, safety googles, and gloves) shall be applied for the execution of this standard operating procedure.

Warning: Boron trifluoride is toxic

## 4 Materials and methods

### 4.1 Reagents

- 4.1.1 n-Hexane; ECD quality
- 4.1.2 Dichloromethane; GC quality
- 4.1.3 Methanol; p.a.
- 4.1.4 Ethyl acetate; p.a.
- 4.1.5 Squalane, purity >99%
- 4.1.6 Glyceryl triheptadecanoate (tri-C17:0), CAS 2438-40-6, purity >99%
- 4.1.7 C7-C40 saturated alkane standard, 1000 µg/ml
- 4.1.8 Palmitic acid methyl ester (C16:0-Me), purity >99%
- 4.1.9 Heptadecanoic acid methyl ester (C17:0-Me), purity >99%
- 4.1.10 Oleic acid methyl ester (C18:1-Me), purity >99%
- 4.1.11 Stearic acid methyl ester (C18:0-Me), purity >99%
- 4.1.12 Boron trifluoride dimethanol complex (20% w/w)
- 4.1.13 Sodium chloride p.a.
- 4.1.14 Solvent mix
  - Mix 1000 ml (680 g) n-hexane (4.1.1) and 200 ml (266 g) dichloromethane (4.1.2)
- 4.1.15 Elution mix
  - Mix n-hexane (4.1.1) and ethyl acetate (4.1.4) at equal volumes
- 4.1.16 Sodium chloride solution saturated
  - Dissolve sodium chloride (4.1.13) in deionised water until saturation
- 4.1.17 Sodium sulphate, anhydrous, granular
- 4.1.18 2,2-Dimethoxypropane purity >98%
- 4.1.19 Toluene, p.a.

### 4.2 Standard solutions

The volumes of both standard and solvent preparations may be adjusted as long as the concentrations do not change.

#### 4.2.1 Solutions for paraffin measurements

- 4.2.1.1 *Squalane internal standard solution (2 mg/ml)*
  - Weigh on a balance with 0.01 mg readability (4.4.5) **100 mg** squalane (4.1.5) into a **50 ml volumetric flask** and fill with n-hexane (4.1.1) up to mark
- 4.2.1.2 *Squalane solution in n-hexane (10 µg/ml)*
  - Pipette with a positive displacement pipette (4.3.4) **5.0 ml** of “Squalane internal standard solution” (4.2.1.1) into a **1000 ml volumetric flask** and fill up to volume with n-hexane (4.1.1).

- 4.2.1.3 *Squalane solution in solvent mix (20 µg/ml)*  
 Pipette with a positive displacement pipette (4.3.4) 10.0 ml of “Squalane internal standard solution” (4.2.1.1) into a **1000 ml volumetric flask** and fill up to volume with the “Solvent mix” (4.1.14) of n-hexane and dichloromethane
- 4.2.1.4 *n-Alkanes stock standard solution (40 µg/ml)*  
 Transfer from each of two ampoules of “C7-C40 saturated alkane standard” (4.1.7) 1000 µl into the same **50 ml volumetric flask**, evaporate the solvent with a stream of nitrogen, and fill then up to mark with the “Squalane solution in n-hexane” (4.2.1.2).
- 4.2.1.5 *n-Alkanes calibration solutions S1 to S10*  
 n-Alkane calibration solutions are prepared by pipetting with a positive displacement pipette (4.3.4) the volumes listed in Table 1 of the “n-Alkanes stock solution” (4.2.1.4) into **10 ml volumetric flasks** and filling up to volume with the “Squalane solution in n-hexane” (4.2.1.2). The nominal standard concentrations are given in the third column of Table 1. The standard concentrations used for the calculation of the calibration functions shall take into account the purity of applied standard solutions, the actual concentration of the stock standard solution (4.2.1.4), as well as any deviation from the preparation scheme.

*Table 1: Preparation of calibration standards and nominal standard concentrations*

	<b>n-Alkanes stock standard solution</b>	<b>C7-C40</b>
	<i>µl</i>	<i>µg/ml</i>
<b>PS1</b>	100	0.4
<b>PS2</b>	200	0.8
<b>PS3</b>	300	1.2
<b>PS4</b>	500	2.0
<b>PS5</b>	1000	4.0
<b>PS6</b>	2000	8.0
<b>PS7</b>	3000	12.0
<b>PS8</b>	5000	20.0
<b>PS9</b>	7000	28.0
<b>PS10</b>	9000	36.0

- 4.2.2 Solutions for fatty acid methyl ester (FAME) measurement
- 4.2.2.1 *Glyceryl triheptadecanoate solution (0.8 mg/ml)*  
 Weigh on a balance with readability 0.01 mg (4.4.5) 20 mg glyceryl triheptadecanoate (4.1.6) into a 25 ml volumetric flask and fill up to mark with n-hexane (4.1.1)
- 4.2.2.2 *Heptadecanoic acid methyl ester (C17:0-Me) stock standard (1 mg/ml)*  
 Weigh on a balance with readability 0.01 mg (4.4.5) 50 mg heptadecanoic acid methyl ester (C17:0-Me) (4.1.9) into a 50 ml volumetric flask and fill up to mark with n-hexane (4.1.1)
- 4.2.2.3 *Oleic acid methyl ester (C18:1-Me) stock standard (10 mg/ml)*  
 Weigh on a balance with readability 0.01 mg (4.4.5) 100 mg of oleic acid methyl ester (C18:1-Me) (4.1.10) into a 10 ml volumetric flask, dissolve and fill up to mark with n-hexane (4.1.1)

- 4.2.2.4** *Palmitic acid methyl ester (C16:0-Me) stock standard (10 mg/ml)*  
 Weigh on a balance with readability 0.01 mg (4.4.5) 100 mg of palmitic acid methyl ester (C16:0-Me) (4.1.8) into a 10 ml volumetric flask, dissolve and fill up to mark with n-hexane (4.1.1)
- 4.2.2.5** *Stearic acid methyl ester (C18:0-Me) stock standard (10 mg/ml)*  
 Weigh on a balance with readability 0.01 mg (4.4.5) 100 mg of stearic acid methyl ester (C18:0-Me) (4.1.11) into a 10 ml volumetric flask, dissolve and fill up to mark with n-hexane (4.1.1)
- 4.2.2.6** *FAME mix standard (each 100 µg/ml)*  
 Pipette with a positive displacement pipette (4.3.4) 500 µl of each of the “Oleic acid methyl ester (C18:1-Me) stock standard” (4.2.2.3), “Palmitic acid methyl ester (C16:0-Me) stock standard” (4.2.2.4), and “Stearic acid methyl ester (C18:0-Me) stock standard” (4.2.2.5) into a 50 ml volumetric flask and fill up to mark with n-hexane (4.1.1).
- 4.2.2.7** *Heptadecanoic acid methyl ester calibration standard (1 µg/ml)*  
 Pipette with a positive displacement pipette (4.3.4) 5000 µl of the “Heptadecanoic acid methyl ester (C17:0-Me) stock standard” (4.2.2.2) into a 25 ml volumetric flask and fill up to mark with n-hexane (4.1.1)
- 4.2.2.8** *FAME calibration solutions*  
 FAME calibration solutions are prepared by pipetting with a positive displacement pipette (4.3.4) the volumes listed in Table 2 of the “FAME mix standard” (4.2.2.6) and 250 µl of the “Heptadecanoic acid methyl ester calibration standard” (4.2.2.7) into 10 ml volumetric flasks and filling up to volume with n-hexane (4.1.1). The nominal standard concentrations are given in Table 2 as well.  
 The standard concentrations used for the calculation of the calibration functions shall take into account the purity of applied standard solutions as well as any deviation from the preparation scheme.

*Table 2: Preparation of FAME calibration standards and nominal standard concentrations*

Standard	Pipetted volumes		Nominal standard concentrations			
	FAME mix standard	C17:0-Me	C16:0-Me	C18:0-Me	C18:1-Me	C17:0-Me
	4.2.2.6	4.2.2.7				
	µl	µl	µg/ml	µg/ml	µg/ml	µg/ml
FS1	100	250	1.0	1.0	1.0	10.0
FS2	200	250	2.0	2.0	2.0	10.0
FS3	300	250	3.0	3.0	3.0	10.0
FS4	500	250	5.0	5.0	5.0	10.0
FS5	750	250	7.5	7.5	7.5	10.0
FS6	1000	250	10.0	10.0	10.0	10.0
FS7	1500	250	15.0	15.0	15.0	10.0
FS8	2000	250	20.0	20.0	20.0	10.0
FS9	2500	250	25.0	25.0	25.0	10.0
FS10	3000	250	30.0	30.0	30.0	10.0

### **4.3 Materials**

- 4.3.1 LC-Si Solid phase extraction cartridges, 500 mg, 3 ml
- 4.3.2 Test tubes with PTFE lined screw caps
- 4.3.3 DB-1HT capillary column (15m x 250 µm x 0.10 µm)
- 4.3.4 Positive displacement pipette with suitable pipette tips: This type of pipette is used for handling of all defined volumes of organic solvents.
- 4.3.5 Pasteur pipettes, 230 mm
- 4.3.6 Suitable autosampler vials with PTFE/butyl rubber lined crimp caps
- 4.3.7 10 ml flat bottom screw cap glass vials with PTFE lined screw caps

### **4.4 Instrumentation**

- 4.4.1 Gas chromatograph with PTV or split/splitless inlet and FID detector or
- 4.4.2 Gas chromatograph with PTV or split/splitless inlet and single quadrupole mass spectrometer
- 4.4.3 Dry block sample heater preferably with N<sub>2</sub> evaporation possibility, compatible with test tubes (4.3.2)
- 4.4.4 Centrifuge, compatible with test tubes (4.3.2)
- 4.4.5 Balance, readability 0.01 mg
- 4.4.6 Shaker suitable for intensive shaking of test tubes (4.3.2) e.g. wrist arm shaker
- 4.4.7 Vortex mixer

#### 4.5 Instrument parameters

Instrument parameters given in the following are indicative. The analyst may chose different parameters provided that the selectivity of the chromatography system is maintained.

##### 4.5.1 Paraffin n-alkanes measurement by GC-FID

Either PTV or split/splitless inlets may be applied.

	Parameter settings		
<b>Carrier gas</b>	Helium constant flow 1.0 ml/min		
<b>Oven:</b>	60 °C for 2.0 minutes Rate Final temp Final time 1 15.00 195 0.00 2 50.00 230 0.00 3 15.00 360 5.00		
<b>PTV inlet</b>	Mode: solvent vent mode Initial temperature: 50°C / 2 min Rate 1: 12°C/min to 240 °C / 0 min Rate 2: 10 °C/min to 340 °C / 1 min		
<b>Split/splitless inlet</b>	Mode: split Initial temperature: 280°C Split ratio: 5:1		
<b>Column</b>	DB-1HT; 15m x 250 µm x 0.10 µm		
<b>Injection volume</b>	1 µL		
<b>Detector</b>	Flame ionization detector Temperature: 350 °C Gases: Air: 400 ml/min Hydrogen: 30 ml/min Nitrogen: 40 ml/min		

4.5.2 Fatty acid methyl ester measurement by GC-FID  
 Either PTV or split/splitless inlets may be applied.

	<b>Parameter settings</b>																
<b>Carrier gas</b>	Helium constant flow 1.0 ml/min																
<b>Oven:</b>	60 °C for 2.5 minutes <table> <thead> <tr> <th></th> <th>Rate</th> <th>Final temp</th> <th>Final time</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>30.00</td> <td>180</td> <td>1.00</td> </tr> <tr> <td>2</td> <td>5.00</td> <td>220</td> <td>0.00</td> </tr> <tr> <td>3</td> <td>30.00</td> <td>360</td> <td>5.00</td> </tr> </tbody> </table>		Rate	Final temp	Final time	1	30.00	180	1.00	2	5.00	220	0.00	3	30.00	360	5.00
	Rate	Final temp	Final time														
1	30.00	180	1.00														
2	5.00	220	0.00														
3	30.00	360	5.00														
<b>PTV inlet</b>	Mode: solvent vent mode Initial temperature: 50°C / 2 min Rate 1: 12°C/min to 240 °C / 0 min Rate 2: 10 °C/min to 340 °C / 1 min																
<b>Split/splitless inlet</b>	Mode: splitless Initial temperature: 280°C Purge time: 1.0 min Purge flow: 25 ml/min																
<b>Column</b>	DB-1HT; 15m x 250 µm x 0.10 µm																
<b>Injection volume</b>	1 µL																
<b>Detector</b>	Flame ionization detector Temperature: 350 °C Gases: Air: 400 ml/min Hydrogen: 30 ml/min Nitrogen: 40 ml/min																

#### 4.5.3 Paraffin n-alkanes measurement by GC-MS

Either PTV or split/splitless inlets may be applied.

	<b>Parameter settings</b>
<b>Carrier gas</b>	Helium constant flow 1.0 ml/min
<b>Oven:</b>	45 °C for 2.5 minutes Rate Final temp Final time 1 15.00 195 0.00 2 50.00 230 0.00 3 10.00 340 5.00
<b>PTV inlet</b>	Mode: solvent vent mode Vent flow : 40 ml/min until 2.0 min Vent pressure 40 kPa Purge flow: 35.0 ml/min until 5.0 min Gas saver flow: 20 ml/min at 8.0 min Initial temperature: 45°C / 2.2 min Rate 1: 12°C/min to 350 °C / 10 min
<b>Split/splitless inlet</b>	Mode: splitless Initial temperature: 300°C Purge time: 1.0 min Purge flow: 25 ml/min
<b>Column</b>	DB-1HT; 15m x 250 µm x 0.10 µm
<b>Injection volume</b>	1 µL
<b>Detector</b>	Single quadrupole mass spectrometer Solvent delay: 6.8 min Interphase temperature: 350 °C Ion source temperature: 230 °C Quadrupole temperature: 150 °C Ionization energy: 70 eV Selected Ion Monitoring (SIM): m/z = <u>57</u> (quantifier ion), 71, and 85 (qualifier ions) Dwell time: 50 ms for each ion

4.5.4 Fatty acid methyl ester measurement by GC-MS  
 Either PTV or split/splitless inlets may be applied.

	<b>Parameter settings</b>																
<b>Carrier gas</b>	Helium constant flow 1.0 ml/min																
<b>Oven:</b>	60 °C for 2.5 minutes <table> <thead> <tr> <th></th> <th>Rate</th> <th>Final temp</th> <th>Final time</th> </tr> </thead> <tbody> <tr> <td>1</td> <td>30.00</td> <td>160</td> <td>1.00</td> </tr> <tr> <td>2</td> <td>5.00</td> <td>200</td> <td>0.00</td> </tr> <tr> <td>3</td> <td>50.00</td> <td>340</td> <td>5.00</td> </tr> </tbody> </table>		Rate	Final temp	Final time	1	30.00	160	1.00	2	5.00	200	0.00	3	50.00	340	5.00
	Rate	Final temp	Final time														
1	30.00	160	1.00														
2	5.00	200	0.00														
3	50.00	340	5.00														
<b>PTV inlet</b>	Mode: solvent vent mode Vent flow : 20 ml/min until 1.6 min Vent pressure 50 kPa Purge flow: 50.0 ml/min until 4.5 min Gas saver flow: 20 ml/min at 5.0 min Initial temperature: 50°C / 1.8 min Rate 1: 12°C/min to 150 °C / 0 min Rate 2: 10 °C/min to 340 °C / 15 min																
<b>Split/splitless inlet</b>	Mode: splitless Initial temperature: 280°C Purge time: 1.0 min Purge flow: 25 ml/min																
<b>Column</b>	DB-1HT; 15m x 250 µm x 0.10 µm																
<b>Injection volume</b>	1 µL																
<b>Detector</b>	Single quadrupole mass spectrometer Solvent delay: 6.8 min Interphase temperature: 350 °C Ion source temperature: 230 °C Quadrupole temperature: 150 °C Ionization energy: 70 eV Scan range: m/z = 45 to m/z = 350 Ions for quantification (underscored) and peak identification: C17:0-Me: m/z= <u>74</u> , 87, 284, 241 C16:0-Me: m/z= <u>74</u> , 87, 270, 227 C18:1-Me: m/z= <u>55</u> , 69, 83, 264 C18:0-Me: m/z= <u>74</u> , 87, 298, 255																

## 5 Sample preparation

**Figure 1** displays the steps of the sample preparation procedure.

### 5.1.1 Sample dissolution and dilution

#### 5.1.1.1 *Sample dissolution*

Approximately 50 mg of the beeswax test sample is weighed on a balance with 0.01 mg readability (4.4.5) into a test tube with screw cap (4.3.2) and dissolved in 5000 µl of the "Squalane solution in solvent mix" (4.2.1.3) (pipetted with a positive displacement pipette (4.3.4)). The mixture is heated to 60 °C until complete dissolution of the beeswax.

#### 5.1.1.2 *Sample dilution*

After completeness of dissolution, the sample solution is cooled down to room temperature and an aliquot of 1000 µl of the concentrated mixture is diluted in a fresh test tube with 4000 µl of the "Squalane solution in solvent mix" (4.2.1.3).

### 5.1.2 Sample fractionation

The first sample preparation step comprises fractionation by solid phase extraction which consists of five steps:

- 5.1.2.1 *Conditioning of the SPE cartridge with 2000 µl n-hexane (4.1.1),*
- 5.1.2.2 *Loading of 500 µL of the diluted sample (5.1.1.2) on the SPE cartridge,*
- 5.1.2.3 *Addition of 250 µl of glyceryl triheptadecanoate solution (4.2.2.1),*
- 5.1.2.4 *Addition of 2000 µl of n-hexane (4.1.1) and collection of the paraffin fraction (F1) in a fresh test tube (4.3.2),*
- 5.1.2.5 *Elution of the stearin/stearic acid fraction with 2500 µl of the "Elution mix" (4.1.15) and collection of this fraction (F2) in a fresh test tube (4.3.2).*

### 5.1.3 Paraffin measurement

Transfer an aliquot of fraction F1 (5.1.2.4) into an autosampler vial and measure the sample by GC-FID or GC-MS applying the parameters specified under 4.5.

#### 5.1.4 Derivatisation of fraction F2

Derivatisation of stearic acid and transesterification of stearin is required for the measurement by gas chromatography with either flame ionisation or mass spectrometric detection. The reaction product is in both cases stearic acid methyl ester.

- 5.1.4.1 Pipette an aliquot of 500 µL of fraction F2 (5.1.2.5) into a fresh test tube (4.3.2) and evaporate at approximately 40 °C under a stream of nitrogen gas to dryness
- 5.1.4.2 Add 250 µL of toluene (4.1.19) to dissolve residue
- 5.1.4.3 Add 1500 µL of BF<sub>3</sub> dimethanol complex derivatization agent (4.1.12) and 50 µL of 2,2-dimethoxypropane (4.1.18) into the test tube and close tightly. The mixture is shaken vigorously on a Vortex mixer (4.4.7).
- 5.1.4.4 Put the test tube at minimum 70 °C for minimum 12 hours on a suitable dry block heater (4.3.2)
- 5.1.4.5 Remove the test tube from the dry block heater and let it cool down to room temperature
- 5.1.4.6 Add 5000 µL of saturated NaCl solution (4.1.16) to the reaction mix, close tightly the test tube and shake thoroughly
- 5.1.4.7 Add 2000 µL of n-hexane (4.1.1) to the test tube and close tightly
- 5.1.4.8 Shake the test tube intensively for 30 minutes by application of a suitable shaker (4.4.6)
- 5.1.4.9 Wait until phase separation is complete. This may be accelerated by centrifugation.
- 5.1.4.10 An aliquot of around 1.5 mL of the n-hexane phase is transferred into a fresh test tube (4.3.2) and a small amount of granular anhydrous sodium sulphate (4.1.17) is added. Shake the tightly closed test tube for about 10 seconds on a Vortex mixer (4.4.7). Separation of solid and liquid layer may be facilitated by centrifugation.

#### 5.1.5 Fatty acid methyl ester measurement

Transfer an aliquot of the n-hexane layer into an autosampler vial and determine fatty acid methyl esters by GC-FID or GC-MS applying the parameters specified under 4.5

## 6 Calculation of results

### 6.1 Paraffin

#### Calibration function

Inject aliquots of calibration standards PS1 to PS10 into the gas chromatograph.

Calculate the area ratio between analyte peaks and the internal standard peak (squalane)

Calculate the ratios between analyte concentrations and the concentration of the internal standard (squalane)

Plot the area ratios against the concentration ratios and determine both the slope of the calibration curve ( $a_{Cal}$ ) and the intercept of the calibration curve ( $b_{Cal}$ ) by linear regression

#### Paraffin content:

$$C_{Analyte} = \left( \frac{A_{Analyte}}{A_{ISTD}} - b_{Cal} \right) * \frac{m_{ISTD}}{a_{Cal} * w * 200}$$

$C_{Analyte}$ : Content of analyte in **mg/g** test sample

$A_{Analyte}$ : Peak area of analyte peak in the test sample

$A_{ISTD}$ : Peak area of internal standard peak in the test sample

$m_{ISTD}$ : Amount in **µg** of internal standard (squalane) contained in 5 ml of solution 4.2.1.3 (nominal 100 µg), which is added in step 5.1.1.1 to the beeswax sample

$a_{Cal}$ : Slope of calibration curve

$b_{Cal}$ : Intercept of calibration curve

w: Sample weight in **g**

Results shall be expressed with one digit after the comma.

## 6.2 Fatty acids

### Calibration function

Inject aliquots of calibration standards FS1 to FS10 into the gas chromatograph.

Calculate the area ratio between analyte peaks and the internal standard peak (heptadecanoic acid methyl ester)

Calculate the ratios between analyte concentrations and the concentration of the internal standard (heptadecanoic acid methyl ester)

Plot the area ratios against the concentration ratios and determine both the slope of the calibration curve ( $a_{Cal}$ ) and the intercept of the calibration curve ( $b_{Cal}$ ) by linear regression

### Fatty acid content:

$$C_{Analyte} = \left( \frac{A_{Analyte}}{A_{ISTD}} - b_{Cal} \right) * \frac{m_{ISTD} * R * X}{a_{Cal} * w * 20}$$

$C_{Analyte}$ : Content of free fatty acid in **mg/g** test sample

$A_{Analyte}$ : Peak area of analyte peak in the test sample

$A_{ISTD}$ : Peak area of internal standard peak in the test sample

$m_{ISTD}$ : Amount in **µg** of internal standard glyceryl triheptadecanoate added in step 5.1.2.3 onto the SPE (nominal 200 µg)

$a_{Cal}$ : Slope of calibration curve

$b_{Cal}$ : Intercept of calibration curve

R: 1.0048 (ratio of molar masses of 3 mol of heptadecanoic acid methyl ester and 1 mol glyceryl triheptadecanoate).

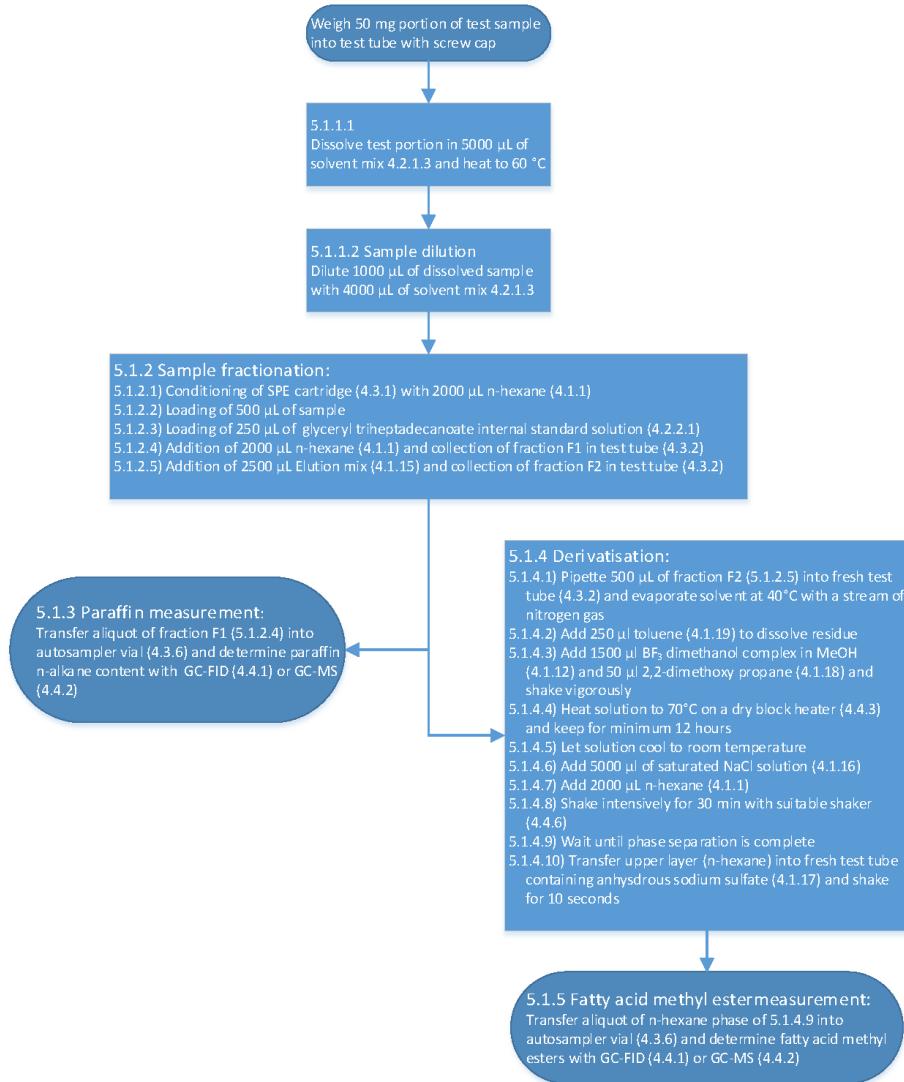
X: Ratio of molar masses of fatty acid to fatty acid methyl ester:

C16:0 / C16:0-Me	0.948
C18:1 / C18:1-Me	0.953
C18:0 / C18:0-Me	0.953

w: Sample weight in g

Results shall be expressed with one digit after the comma.

**Figure 1: Flow chart of sample preparation procedure**



### Annex 3. Sample description

Number	Type	Form	Colour	Country of origin	Label declaration
1	Beeswax	block	yellow	FR	
2	Beeswax	candle	yellow	-	
3	Beeswax	pastilles	orange	-	pure beeswax
4	Beeswax	pastilles	white	-	<i>Cera alba</i> pure
5	Beeswax	pastilles	yellow	-	
6	Paraffin	pastilles	white		100 % pure paraffin
7	Stearic acid wax	powder	white		
8	Beeswax	block	dark yellow	-	100 % natural beeswax
9	Beeswax	pastilles	yellow	-	100 % natural
10	Beeswax	pastilles	white	-	natural wax
11	Beeswax	pastilles	white	-	100 % natural beeswax
12	Beeswax	pastilles	yellow	-	100 % natural
13	Beeswax	block	yellow	CN	
14	Beeswax	pastilles	dark yellow	-	100 % natural beeswax
15	Beeswax	pastilles	light yellow	-	100 % pure beeswax
16	Beeswax	pastilles	white	-	<i>Cera alba</i> , organic
17	Beeswax	candle	dark yellow	-	
18	Beeswax	block	yellow	CN	
19	Beeswax	block	yellow	-	natural pure beeswax
20	Beeswax	pastilles	yellow	-	100 % natural
21	Beeswax	pastilles	yellow	-	100 % natural beeswax
22	Beeswax	pastilles	yellow	-	<i>Cera alba</i> , pure natural
23	Beeswax	pastilles	yellow	-	100 % <i>cera alba</i> , <1% paraffin, <2% stearic acid (GC)
24	Beeswax	foundation	dark yellow	ES	
25	Beeswax	pastilles	dark yellow/brown	-	100 % <i>cera flava</i>
26	Beeswax	foundation	dark yellow/brown	-	100 % beeswax
27	Beeswax	foundation	light yellow	-	
28	Beeswax	foundation	yellow	CN	pure beeswax
29	Beeswax	pastilles	yellow	-	
30	Beeswax	block	yellow	-	
31	Beeswax	block	white	-	
32	Beeswax	block	yellow	-	
33	Beeswax	pastilles	yellow	-	
34	Beeswax	block	orange	-	
35	Beeswax	block	light brown	AR	crude beeswax
36	Beeswax	block	dark brown	Africa	crude beeswax
37	Beeswax	block	yellow	CN	crude beeswax

38	Beeswax	pastilles	light yellow	NL	refined beeswax
39	Beeswax	block	yellow	CN	crude beeswax
40	Beeswax	block	yellow	CN	crude beeswax
41	Beeswax	block	yellow	CN	crude beeswax
42	Beeswax	block	dark brown	ZM	crude beeswax
43	Beeswax	block	brown	ET	crude beeswax
44	Beeswax	block	dark brown	ET	crude beeswax
45	Beeswax	block	yellow	AR	crude beeswax
46	Beeswax	block	yellow	AR	crude beeswax
47	Beeswax	block	yellow	AR	crude beeswax
48	Beeswax	block	yellow	AU	crude beeswax
49	Beeswax	block	yellow	AU	crude beeswax
50	Beeswax	block	yellow	AU	crude beeswax
51	Wax	block	white	-	Microcrystalline wax, low melting point
52	Stearic acid	powder	white	-	
53	Paraffin	block	white	-	
55	Beeswax	candle	yellow	CZ	
56	Beeswax	honeycomb	yellow	-	
57	Beeswax	honeycomb	dark yellow	-	
60	Beeswax	block	yellow	-	
61	Beeswax	block	yellow	-	
62	Beeswax	candle	yellow	-	
63	Beeswax	candle	light yellow	-	
64	Beeswax	candle	light yellow	-	
65	Beeswax	pearls	yellow	-	
66	Beeswax	foundation	yellow	-	
67	Beeswax	foundation	white	-	
68	Beeswax	foundation	yellow	-	
69	Beeswax	thick candles	yellow	-	
70	Beeswax	foundation	white	-	
71	Beeswax	foundation	yellow	-	
72	Beeswax	foundation	light yellow	-	
73	Beeswax	foundation	yellow	-	
74	Beeswax	block	yellow	-	
75	Beeswax	foundation	yellow	-	
76	Beeswax	honeycomb	yellow	-	
77	Beeswax	foundation	yellow	AT	
78	Beeswax	honeycomb	yellow	AT	
79	Beeswax	foundation	yellow	-	
80	Beeswax	block	yellow	-	
81	Beeswax	block	yellow	-	
82	Beeswax	honeycomb containing honey	yellow	GR	

83	Beeswax	honeycomb containing honey	black	GR	
84	Beeswax	honeycomb containing honey	yellow	GR	
85	Beeswax	honeycomb containing honey	brown	BE	
86	Beeswax	honeycomb containing honey	yellow/gold	DE	
87	Beeswax	honeycomb containing honey	gold	DE	
88	Beeswax	honeycomb containing honey	gold	BG	
89	Beeswax	honeycomb containing honey	gold	IT	
90	Beeswax	honeycomb containing honey	yellow/gold	DE	
91	Beeswax	honeycomb containing honey	yellow/gold	DE	
92	Beeswax	honeycomb containing honey	yellow/gold	DE	
93	Beeswax	honeycomb containing honey	gold	BG	
94	Beeswax	honeycomb containing honey	yellow/gold	HU	
95	Beeswax	honeycomb containing honey	yellow/gold	HU	
96	Beeswax	honeycomb containing honey	gold	DE	
97	Beeswax	honeycomb containing honey	gold	DE	
98	Beeswax	honeycomb containing honey	gold	DE	
99	Beeswax	honeycomb containing honey	gold	NL	
100	Beeswax	honeycomb containing honey	gold	NL	
101	Beeswax	candle	gold	NL	
102	Beeswax	candle	gold	NL	
103	Beeswax	honeycomb containing honey	gold	BE	
104	Beeswax	honeycomb containing honey	brown	NL	
105	Beeswax	honeycomb containing honey	gold	NL	
106	Beeswax	honeycomb containing honey	gold	TR	

107	Beeswax	honeycomb containing honey	gold	TR	
108	Beeswax	honeycomb containing honey	gold	TR	
109	Beeswax	honeycomb containing honey	yellow/gold	BG	
110	Beeswax	honeycomb containing honey	yellow/gold	BG	
111	Beeswax	honeycomb containing honey	yellow/gold	BG	
112	Beeswax	honeycomb containing honey	yellow/gold	BG	
113	Beeswax	honeycomb containing honey	gold	AT	

## **GETTING IN TOUCH WITH THE EU**

### **In person**

All over the European Union there are hundreds of Europe Direct centres. You can find the address of the centre nearest you online ([european-union.europa.eu/contact-eu/meet-us\\_en](http://european-union.europa.eu/contact-eu/meet-us_en)).

### **On the phone or in writing**

Europe Direct is a service that answers your questions about the European Union. You can contact this service:

- by freephone: 00 800 6 7 8 9 10 11 (certain operators may charge for these calls),
- at the following standard number: +32 22999696,
- via the following form: [european-union.europa.eu/contact-eu/write-us\\_en](http://european-union.europa.eu/contact-eu/write-us_en).

## **FINDING INFORMATION ABOUT THE EU**

### **Online**

Information about the European Union in all the official languages of the EU is available on the Europa website ([european-union.europa.eu](http://european-union.europa.eu)).

### **EU publications**

You can view or order EU publications at [op.europa.eu/en/publications](http://op.europa.eu/en/publications). Multiple copies of free publications can be obtained by contacting Europe Direct or your local documentation centre ([european-union.europa.eu/contact-eu/meet-us\\_en](http://european-union.europa.eu/contact-eu/meet-us_en)).

### **EU law and related documents**

For access to legal information from the EU, including all EU law since 1951 in all the official language versions, go to EUR-Lex ([eur-lex.europa.eu](http://eur-lex.europa.eu)).

### **Open data from the EU**

The portal [data.europa.eu](http://data.europa.eu) provides access to open datasets from the EU institutions, bodies and agencies. These can be downloaded and reused for free, for both commercial and non-commercial purposes. The portal also provides access to a wealth of datasets from European countries.

# Science for policy

The Joint Research Centre (JRC) provides independent, evidence-based knowledge and science, supporting EU policies to positively impact society



**EU Science Hub**

[joint-research-centre.ec.europa.eu](http://joint-research-centre.ec.europa.eu)

- [@EU\\_ScienceHub](#)
- [EU Science Hub - Joint Research Centre](#)
- [EU Science, Research and Innovation](#)
- [EU Science Hub](#)
- [@eu\\_science](#)



Publications Office  
of the European Union