Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 67 (2006) 2580–2589
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Raman spectroscopic monitoring of *Lactarius* latex

Kris De Gussem [a,*], Annemieke Verbeken [b], Peter Vandenabeele [a], Joke De Gelder [a], Luc Moens [a]

[a] *Department of Analytical Chemistry, Ghent University, Proeftuinstraat 86, B-9000 Gent, Belgium*
[b] *Department of Biology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium*

Received 23 June 2006; received in revised form 4 September 2006
Available online 16 October 2006

Abstract

Lactarius is a genus of Basidiomycotina with mainly agaricoid representatives, which are characterised by the excretion of a typical milky fluid. In particular, the colour, changes and taste of this latex-like milk are often used as a taxonomically important character. When it is exuded several chemical reactions occur. To date, NMR spectroscopy is generally used for chemical investigation of this latex. However, as a vibrational spectroscopic technique Raman spectroscopy has several advantageous properties for this type of research.

The aim of this study is to investigate whether Raman spectroscopy can be used as an alternative analytical technique to monitor the chemical reactions in *Lactarius* latex. Therefore, this paper presents the first Raman spectra of *Lactarius* latex and provides an interpretation of the Raman bands that are present. *L. lacunarum* latex spectra are thoroughly investigated by 2D correlation analysis and are compared with latex spectra of other species (*L. chrysorrheus*, *L. deterrimus*, *L. fluens*, *L. glyciosmus* and *L. salmonicolor*).

Keywords: *Lactarius*; *Russulales*; Basidiomycotina; Latex; Chemical analysis; 2D correlation; Raman spectroscopy

1. Introduction

For centuries, the systematic study of higher fungi has mainly been based on macromorphological (e.g. dimensions, colour, type and shape of sporophores) and various microscopical characters (e.g. the arrangement of hyphae in different tissues and the form of structures dedicated to sexual and asexual reproduction) (Carlile and Watkinson, 1994). One particular character of the genus *Lactarius* is that all species of this genus exude a typical milky fluid when their basidiocarps are broken. Depending on the species, this milky fluid or latex may taste mild or acrid and may be white or coloured (orange, yellow…) or may have a changing colour, thus providing important taxonomical information (Heilmann-Clausen et al., 1998). The latex is produced in the lactiferous hyphal system, consisting of rather broad hyphae present in the whole basidiocarp. The latex is a complex mixture of metabolites and is mainly of sesquiterpenoid origin. Interestingly, the compounds in the latex of a *Lactarius* species are thought to be formed from one single fatty-acid precursor in the latex (Hansson et al., 1995), although this precursor may vary for different species. It was stated that the precursors can be divided into different groups: chromene derivatives are detected in *L.* section *Plinthogali*; fatty acid esters of velutinal are found as precursors in another large group (*L.* section *Albati* and *L.* section *Lactarius*); in the subgenus *Piperites*, guaiane sesquiterpenes are found in *L.* section *Deliciosi*, while other sections in this subgenus are not profoundly investigated. All precursors are inactive forms of metabolites and are stored in the undamaged lipid layers of cell membranes (Daniewski et al., 1995).

After release from the injured fruit bodies, several enzymatic induced transformations occur in the latex (Clericuzio and Sterner, 1997; Daniewski et al., 1996; Hansson et al., 1995). These reactions include amongst others oxidation, lactonisation, aldol condensation and formation or

* Corresponding author. Tel.: +32 9 2646609; fax: +32 9 2646699.
E-mail address: Kris.DeGussem@UGent.be (K. De Gussem).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.09.006

breaking of carbon–carbon double bonds. For example, guaiane sesquiterpenoids frequently undergo colour changes due to enzymatic induced reactions, while chromene derivatives may turn reddish and bitterly acrid (Hansson et al., 1995). The produced metabolites may be interesting for the pharmaceutical industry, such as velutinal based sesquiterpenes that often have 1,4-dialdehyde functional groups. These are frequently bioactive with either antimicrobial, cytotoxic, antifeedant or mutagenic activity (Gamba-Invernizzi et al., 1993; Hansson et al., 1995; Suortti et al., 1983). As an example, Daniewski et al. (1995) have observed that isovelleral, found in e.g. *L. vellereus*, possesses important bacteriostatic and mutagenic activities against storage pests such as *Tribolium confusum*. It has therefore been suggested that the latex is part of a chemical defence system against parasites and predators (Sterner et al., 1985b). This way, the latex can serve as a storage medium for labile compounds (Camazine and Lupo, 1984). While colour changes may frighten parasites, acrid tastes are often avoided by attacking organisms.

Analysis of the chemical composition as well as chemical reaction monitoring was performed using ^{1}H and ^{13}C NMR spectroscopy. In these studies (Bergendorff and Sterner, 1988; Clericuzio and Sterner, 1997; Daniewski et al., 1995, 1996; De Bernardi et al., 1993; Gamba-Invernizzi et al., 1993; Garlaschelli et al., 1994; Hansson et al., 1995; Sterner et al., 1985a,b; Suortti et al., 1983), chromatographic techniques such as thin layer chromatography (TLC) or high performance liquid chromatography (HPLC) were also used for separation of the different sesquiterpenoid compounds. Individual compounds were characterised and quantified in order to analyze the chemical pathway and quantify the reaction rates. This way the chemical basis of taxonomic characters could be deduced and chemotaxonomic characters were used to support taxonomic investigations. Clericuzio and Sterner (1997) also performed molecular mechanics calculations in their study, to predict the structure of stable conformations of the sesquiterpenoids. In addition, De Bernardi et al. (1992) synthesized and characterised the different compounds and completely determined the reaction pathway of *L. fuliginosus* and *L. picinus*.

In previous studies, a time consuming and demanding sample preparation was often necessary prior to analysis and therefore the chemical composition in *Lactarius* latex was monitored discontinuously. Raman spectroscopy is an analytical technique which possesses some advantageous properties for reaction monitoring (McCreery, 2000) as this vibrational spectroscopic technique offers fast and non-destructive analysis of the chemical composition, while only a limited sample preparation is necessary. In addition, the Raman spectrum of water is weak and barely interferes with the measurements. Raman bands of most chemical compounds are narrow, which is advantageous for the analysis of complex mixtures. A disadvantage of Raman spectroscopy in comparison with other studies is the fact that exact structure elucidation is not possible. Therefore, Raman spectroscopy should be regarded as a complementary technique to NMR- and mass spectroscopy. Due to instrumental progression in the last two decades, Raman spectroscopy became available for the demanding analyses of biological materials and liquids, such as *Lactarius* spores (De Gussem et al., 2005).

Raman spectroscopy is used in this study to investigate *Lactarius* latex and its reactions. Chemical reactions are time-related phenomena and so the Raman spectra of a reaction mixture reflect the chemical composition at each moment. 2D correlation analysis is a mathematical technique which was first developed by Noda et al. in 1986 for infrared spectroscopy and was generalized and extended by several authors (Noda and Ozaki, 2004). 2D correlation analysis is applicable to all kinds of spectroscopic data and is especially suitable for the analysis of time-correlated phenomena or correlations between samples. In general, 2D spectra are represented as contour plots, from which the symmetry axis, also called the power spectrum, shows time-correlated spectral features (e.g. Raman bands). Off-axis signals indicate whether changes in one spectral feature correlate with changes in other features. A positive off-axis peak indicates that two features increase or decrease together (positive correlation), while a negative 2D synchronous peak indicates that one feature increases while another one decreases in intensity (negative correlation). Next to these 2D synchronous spectra, 2D correlation analysis also results in 2D asynchronous spectra that are useful to analyse whether intensity changes in spectra are a result of the same reaction (features change in-phase, at the same time) or not (features change out-of-phase, at different moments).

The aim of this study is to investigate whether Raman spectroscopy could be used to monitor the chemical reactions in *Lactarius* latex after its release from the basidiocarp. This project is a first step in the analysis of chemical reactions of *L.* latex using Raman spectroscopy. This paper presents Raman spectra of *Lactarius* latex, along with the attribution of some Raman bands to chemical compounds. The chemical reactions of *L. lacunarum* latex are analysed in detail by 2D correlation analysis and its spectral changes are compared with those of other species.

2. Results and discussion

De Bernardi et al. (1993) reported different types of sesquiterpenoid precursors in *Lactarius* latex and our data of *L. lacunarum* latex seem to correspond with a fenolic fatty acid ester (molecule 3 in De Bernardi et al. (1993)), which was found in *L. fuliginosus* (*L.* section *Plinthogali*). Based on the observed reaction pattern and a band assignment (Socrates, 2001) of the Raman bands affected by the chemical reactions, the precursor in *L. lacunarum* latex (*L.* section *Russularia*) is proposed to be similar to the molecule in Fig. 1.

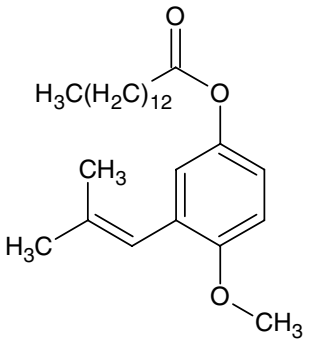

Fig. 1. Possible precursor of the bioactive compound in *Lactarius lacunarum* latex.

2.1. Chemical reaction monitoring

Fig. 2 clearly illustrates the chemical changes in *Lactarius* latex as observed in the Raman spectra. Background subtracted *L. lacunarum* latex spectra, which are collected at different moments after exudation, are presented. In these spectra, many bands can be observed which change in intensity over time and the molecular functional groups responsible for these Raman bands are thus affected by these chemical changes.

Many bands, e.g. at 907, 1004, 1063, 1092, 1130, 1420, 1564, 1592 and 1698 cm^{-1} appear during the experiment or are characterised by an increasing intensity over time. These phenomena are characteristic for molecular groups which are formed. In addition, Raman bands below 550 cm^{-1}, between 750 and 900 cm^{-1} and between 940 and 1000 cm^{-1} have generally a decreasing intensity. These signals are indicative for functional groups which disappear due to reactions. Next to the clearly appearing and disappearing Raman bands, there are different Raman bands subject to band shifting. Examples of shifting bands can be observed at 590, 810, 1171, 1300, 1335, 1378, 1445 and 1457 cm^{-1} which are found at the end of the experiment at 596, 814, 1177, 1296, 1340, 1372, 1441 and 1462 cm^{-1}, respectively. These shifts are generally due to slight modifications of the functional group or to modifications in the near environment of the functional group responsible for the Raman band.

Based on different experiments with *L. lacunarum*, we conclude that the occurring reactions are reproducible, although reaction rates may vary from run to run as well as from specimen to specimen due to factors such as the age of the collected specimens (De Bernardi et al., 1993). Reactions in *L. lacunarum* latex are completed in 12–15 min, after which only minor differences between spectra due to small inhomogenities in the latex can be observed. On a longer timescale (>2 h), the fluorescence background rises, which can be due to polymerisation as observed by Gamba-Invernizzi et al. (1993) for farnesane sesquiterpenes in *L. porninsis*.

2.2. 2D correlation analysis of Lactarius lacunarum latex

2D correlation analysis was performed on the latex spectra in order to study spectral changes due to the chemical transformations in the latex. It is known that 2D correlation spectra are dependant on the mathematical preprocessing used (Noda and Ozaki, 2004). It was observed that the main factor leading to uninformative spectral changes was a drift of the fluorescence background. Lieber and Mahadevan-Jansen (2003) reported that often the fluorescence background of biological samples can be modelled mathematically by fitting a fifth-order

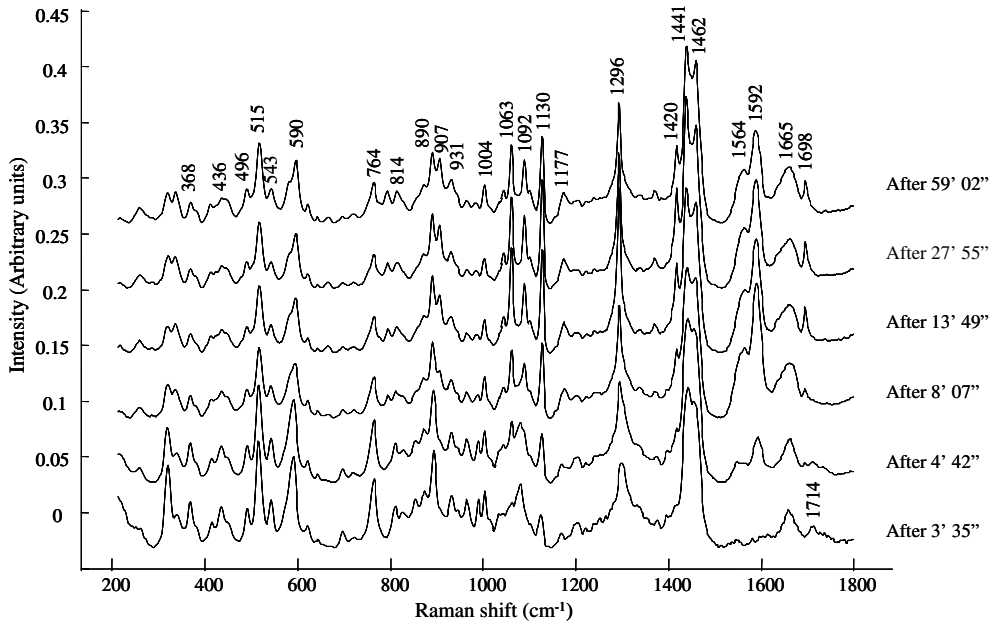

Fig. 2. Overview background subtracted *Lactarius lacunarum* latex spectra, including positions of the discussed signals (when a Raman band shifts, its position in the end is given).

polynomial through the spectra. The fluorescence background was thus subtracted by using an iterative polynomial fitting routine (fifth-order, 200 iterations). In addition to background subtraction, we found that by using mean-centred spectra, the chemical modifications were more clearly visible. Normalisation pre-treatment was not used, since this occasionally can introduce artefacts due to the formation of intense Raman bands for some latexes. Since dispersive Raman spectra may also contain spikes, spectra containing spikes were not included in the analysis and therefore 2D correlation analysis for unevenly spaced data was performed. Typically 3 spectra out of 50 had to be removed.

Fig. 3a shows a contour plot of the 2D synchronous signal of *L. lacunarum* latex. This plot shows several distinct features on its symmetry axis, which indicate the position of the Raman bands affected by the chemical changes. On this power spectrum presented in Fig. 4, one finds the time-correlation of the spectral changes of which the intensities are proportional to the time-correlation. The contours in Fig. 3a show large intensity changes in Raman bands at 1130, 1296, between 1420 and 1470 cm^{-1} and at 1592 cm^{-1}. Most bands are well resolved and can be attributed to single functional groups, except for the broad feature between 1630 and 1690 cm^{-1}. As off-axis features in the 2D synchronous spectrum indicate which chemical

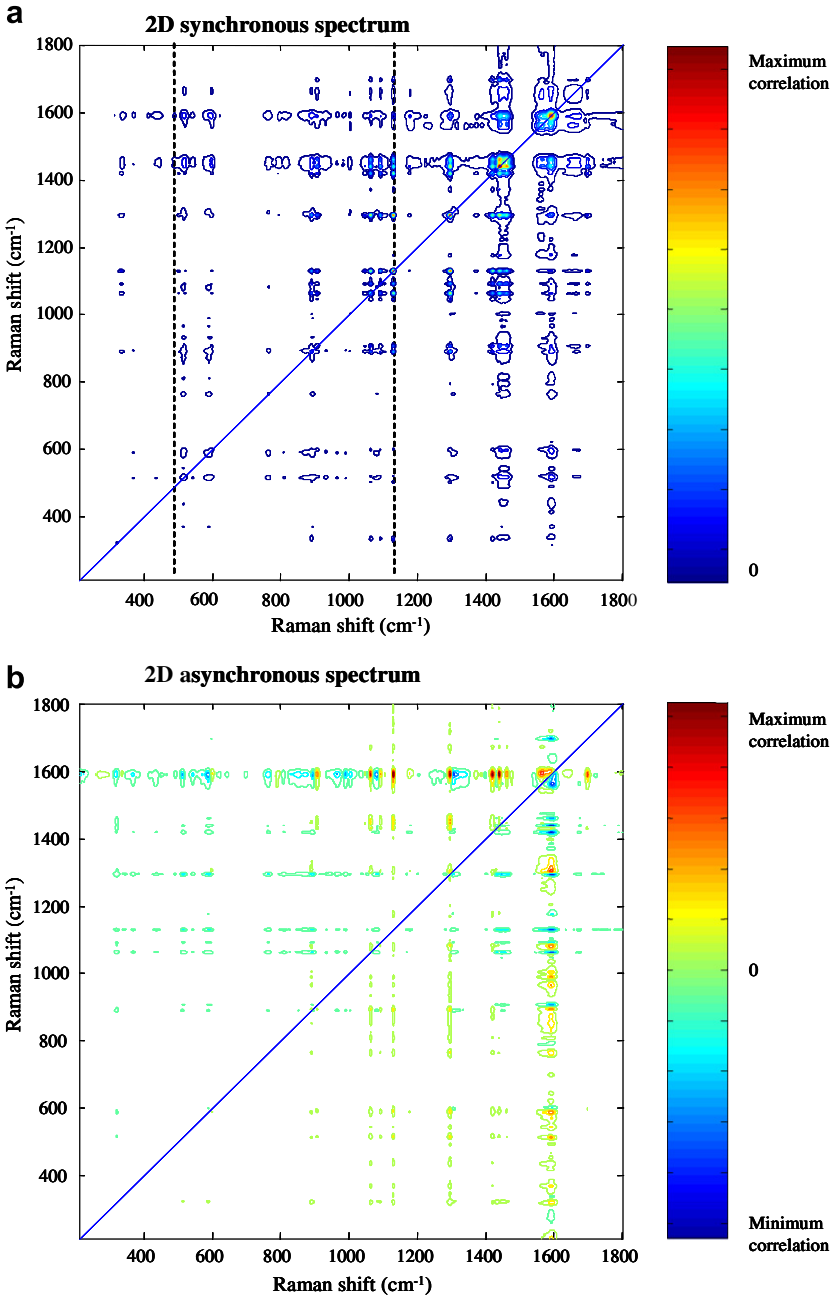

Fig. 3. 2D synchronous (a) and asynchronous (b) spectrum of *Lactarius lacunarum* latex.

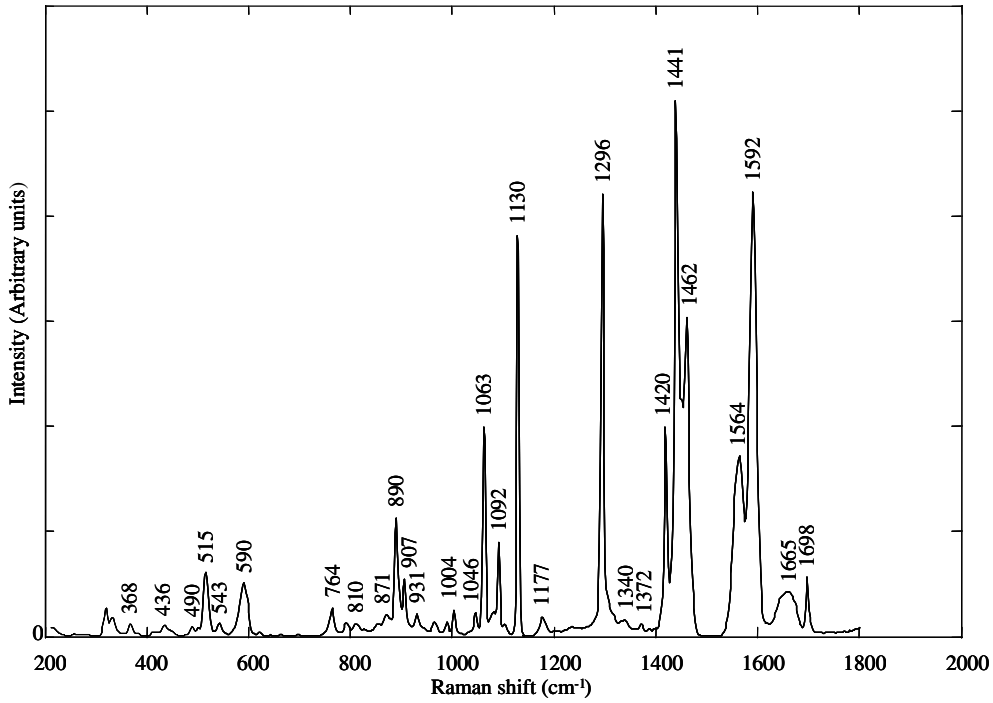

Fig. 4. Symmetry axis signal of the 2D synchronous spectrum (power spectrum) of *Lactarius lacunarum* latex.

modifications are related to each other, careful examination may explain the shape of the feature between 1630 and 1690 cm^{-1}. Unfortunately, the contours of the features in this range are not very detailed. The individual latex spectra however reveal that there is a feature near 1660 cm^{-1}, which seems to be slightly increasing over time (and hence signals appear around 1660 cm^{-1} in the 2D spectra). Close to this Raman band, shoulders appear near

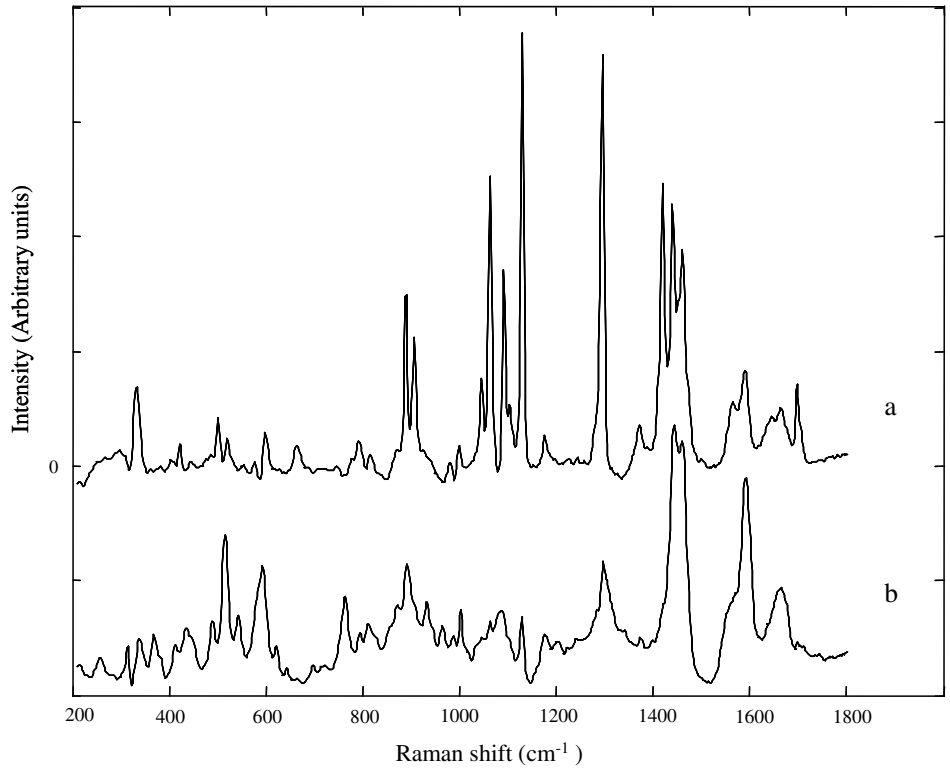

Fig. 5. (a) Slice spectrum at 1130 cm^{-1} and (b) 6× magnified slice spectrum at 490 cm^{-1}, offset of half the maximum correlation at 1130 cm^{-1}.

1636–1640 cm^{-1} and around 1672 cm^{-1}. As a result, at the end of the experiment a broad continuous feature between 1630 and 1690 cm^{-1} is observed.

The synchronous slice spectrum for each frequency gives additional information on the reaction pathway. Fig. 5 presents the slice spectra at 490 and 1130 cm^{-1} as represented by the dashed lines in Fig. 3a. The Raman band at 490 cm^{-1} is not strongly time-correlated, while the correlation plot of the Raman band at 490 cm^{-1}, shown in Fig. 5b, thus consists of broader and less intense signals than the correlation plot of the Raman band at 1130 cm^{-1}. Because of the intensity and the presence of well-resolved signals, 1130 cm^{-1} is therefore chosen as an example for the examination of the off-axis features of the 2D synchronous plot. For example, the positive feature at 1063 cm^{-1} and 1130 cm^{-1} indicates that the Raman bands at 1063 and 1130 cm^{-1} correlate positively. A further signal is found at 1092 cm^{-1} and 1130 cm^{-1} revealing that the Raman band at 1130 cm^{-1} is not only positively correlated with the Raman band at 1063 cm^{-1} but also with the Raman band at 1092 cm^{-1} as well. Further along the line at 1130 cm^{-1}, many more signals are found at the position of Raman bands affected by the chemical reactions. Moreover, when the whole 2D synchronous plot of *L. lacunarum* latex is taken into account, it is clear that the spectral changes in most of the Raman bands are related. This indicates that there occur only a few chemical reactions in the latex. As a result, the most important information for the 2D synchronous spectrum can be drawn from the power spectrum given in Fig. 4.

Table 1 lists the Raman bands affected by the chemical reactions in the *L. lacunarum* latex. As can be seen, the majority of Raman bands higher than 1000 cm^{-1} have an increasing intensity, while in general Raman bands below 1000 cm^{-1} have a decreasing intensity. Of particular interest are the Raman bands at 1063, 1092, 1130 and 1296 cm^{-1}. This is a characteristic pattern for the formation of saturated fatty acids, as the 1296 cm^{-1} band is characteristic for CH-bending vibrations of saturated alkylchains. In addition, it is known from the literature

Table 1
Symmetry axis signal of the 2D synchronous spectrum of *Lactarius lacunarum* latex: tentative assignment of time-correlating Raman bands (Socrates, 2001)

Frequency	Time correlation[a]	Reaction sequence[b]	Band assignment
368	−	4	
436	−	4	
490	−	4	
515	−	4	
543	−	4	Substituted aromatic rings
590	S	4	Substituted aromatic rings
764	−	4	
792	+	1	
810	S	4	
871	−	4	
890	−	4	Out-of-plane δ(CH)-bending vibrations of meta- or trisubstituted aromatic ring with ester substituent
907	+	1	Out-of-plane δ(CH)-bending vibrations of aromatic ring with alcohol substituent
931	+		
965	−	4	
990	−		
1004	+	4	CH in-plane deformation of ortho, meta and trisubstituted aromatic rings
1046	+		CH in-plane deformation of ortho, meta and trisubstituted aromatic rings
1063	+	1	Alkylchain of myristic acid
1082	−	4	
1092	+	1	Alkylchain of myristic acid
1130	+	1	Alkylchain of myristic acid
1177	S	2	
1296	s	1	δ(CH) bend of saturated fatty acids
1340	S	4	
1372	s	1	Myristic acid
1420	+	1	$\delta(CH_2)$, $\delta(CH_3)$ bend
1441	s	1	$\delta(CH_2)$, $\delta(CH_3)$ bend
1462	S	1	$\delta(CH_2)$, $\delta(CH_3)$ bend
1564	+	2	ν(C=C) stretch of aromatic rings
1592	+	3	ν(C=C) stretch of aromatic rings
1636-1640	+	2	
1665	+	2	ν(C=C) stretch of phenols and trialkylalkenes
1672	+	3	ν(C=C) stretch of phenols
1698	+	1	

[a] +, band shows an increasing intensity over time; −, band shows a decreasing intensity over time; s, in addition to intensity differences, the Raman band is shifted to lower wavenumbers; S, in addition to intensity differences, the Raman band is shifted to higher wavenumbers.
[b] Equal numbers indicate that spectral changes occur simultaneously.

that precursors of the active terpenes are stored in the latex as esters of fatty acids, including stearic acid, oleic acid, linoleic acid and palmitic acid esters (Hansson et al., 1995; Clericuzio and Sterner, 1997). As an example, stearoylvelutinal was reported as the fatty acid precursor in *L. vellereus* and *L. bertillonii*. The Raman band positions of stearic acid (18C-atoms in alkylchain) correspond with the 1063 and the 1130 cm^{-1} Raman band. However, no intense Raman band of stearic acid can be observed at 1100 cm^{-1} in *L. lacunarum* latex spectra. This is a clear indication of the absence of stearic acid as the protecting fatty acid. Instead, the Raman bands at 1063, 1092 and 1130 cm^{-1} are representative of myristic acid (14C-atoms in alkylchain).

Next to the Raman bands between 1060 and 1130 cm^{-1} several Raman bands are affected by chemical transformations. They include the typical Raman bands of functional groups of aromatic molecules at 543, 590, 890, 907, 1004, 1046, 1564, 1592, 1665 and 1672 cm^{-1}. The Raman bands at 907, 1665 and 1672 cm^{-1} are characteristic of substituted phenols and show an increasing intensity. Around 1714 cm^{-1} a very weak Raman band, characteristic of ester groups, is present at the beginning of the experiment. It disappears completely after a few minutes (not visible in the 2D spectra), while a band at 1698 cm^{-1} is formed. Although the signal at 1698 cm^{-1} cannot be assigned unambiguously, it is probably due to the formation of a COOH group. Because, besides free phenols, myristic acid is formed, it can be considered that an ester splitting reaction of an aromatic terpenoid precursor occurs.

Fig. 3b presents the asynchronous contour plot corresponding with Fig. 3a. This asynchronous 2D spectrum gives time related information and can be used to reveal the sequence of the spectral changes. Fig. 3b shows its highest intensity signal around 1590 cm^{-1}, indicating large spectral differences in this region, which occur out-of-phase compared to the other intense Raman bands.

More careful inspection of the signals in the asynchronous 2D plot, at the frequencies given in Table 1, reveals that these signals can be considered as 4 distinct groups and a few loosely related signals. It can be concluded that the spectral changes of the frequencies in each different group occur simultaneously and hence these groups represent different chemical reactions. The first group of Raman bands is formed by the signals at 792, 907, 1063, 1092, 1130, 1296, 1372, 1420, 1441, 1462 and 1698 cm^{-1}. These frequencies correspond with the enzymatic induced ester splitting reaction, including related structural changes, of an aromatic compound and the formation of myristic acid. The second group is formed by the signals at 1177, 1564, 1636 and 1665 cm^{-1} which are a result of chemical reactions of the substituents of aromatic rings. Raman spectroscopy, however, is not product specific as the signal around 1665 cm^{-1} could also result from reactions at double bounds. The third group of signals is formed by the Raman bands at 1592 and 1672 cm^{-1} and is caused by chemical transformations of phenols and double bounds. The fact that these Raman bands show an increasing intensity might indicate the formation of a phenol group, originating from, e.g. the splitting of an ether group. The fourth group of closely related Raman bands is composed of the bands at 368, 436, 490, 515, 543, 590, 764, 810, 871, 890, 965, 1004, 1082 and 1340 cm^{-1}. These bands are in general only slightly time-correlated, indicative for some minor structural changes. They are characterised by a decreasing intensity or a shift in their Raman wave numbers. The high intensity of the Raman band at 890 cm^{-1} is the only exception and is currently under investigation. By using the signs of the asynchronous features, it can be concluded that the chemical reactions in *L. lacunarum* latex start with an ester splitting reaction (group 1 of Raman bands). This reaction is followed by chemical transformations of the terpene's aromatic ring (group 2 and/or group 3) and a double bond (group 3). Finally, reactions are slowing down and are eventually terminated (group 4).

2.3. Explorative inter-species comparison of Lactarius latex Raman spectra

The chemical composition of *Lactarius* latex and its relationship with taxonomic grouping are subject to investigation. The majority of research projects concentrated on species with coloured latex. *L. lacunarum* has white latex and the only observable change is a slow yellow staining when it is brought on a white handkerchief.

It can be noticed that in investigated cases, *Lactarius* latex spectra are similar. The reaction affected Raman bands of *L. lacunarum* (*L.* section *Russularia*) and *L. glyciosmus* (*L.* section *Colorati*) therefore give rise to similar 2D patterns (Fig. 6b and c). Taking repeated measurements into account, a notorious difference however, is the shape of the 1665 cm^{-1} Raman band which is well resolved in the case of *L. glyciosmus*. Further spectral differences are shifts in the 515 and 590 cm^{-1} bands of *L. lacunarum* latex, which are, respectively, found at 517 and 596 cm^{-1} in the case of *L. glyciosmus*. In addition, signals at 1177, 1564 and 1592 cm^{-1} are absent in the 2D contour plots of *L. glyciosmus*.

The most obvious spectral difference between *L. lacunarum* latex and *L. fluens* (*L.* section *Glutinosi*, Fig. 6d) is the formation of a small Raman band at 1103 cm^{-1} instead of a larger Raman band at 1092 cm^{-1}, which is a clear indication of the absence of myristic acid as protecting fatty acid of the terpenoid precursor. Furthermore, a high intensity band at 1666 cm^{-1} is observed in the latex of *L. fluens*, indicative for a higher amount of reacting and substituted double bonds in the latex molecules. These spectral differences are also macroscopically clearly visible: while *L. lacunarum* latex is white, *L. fluens* has white latex, which is slowly turning brownish. Nevertheless, the aromatic content of *L. fluens* latex is confirmed by Raman bands at 790, 891, 907, 1003 and 1043 cm^{-1}, although with a different intensity compared to *L. lacunarum*.

L. chrysorrheus (*L.* section *Zonarii*) latex has a white colour when exuded, but turns yellow after a while. 2D spectra

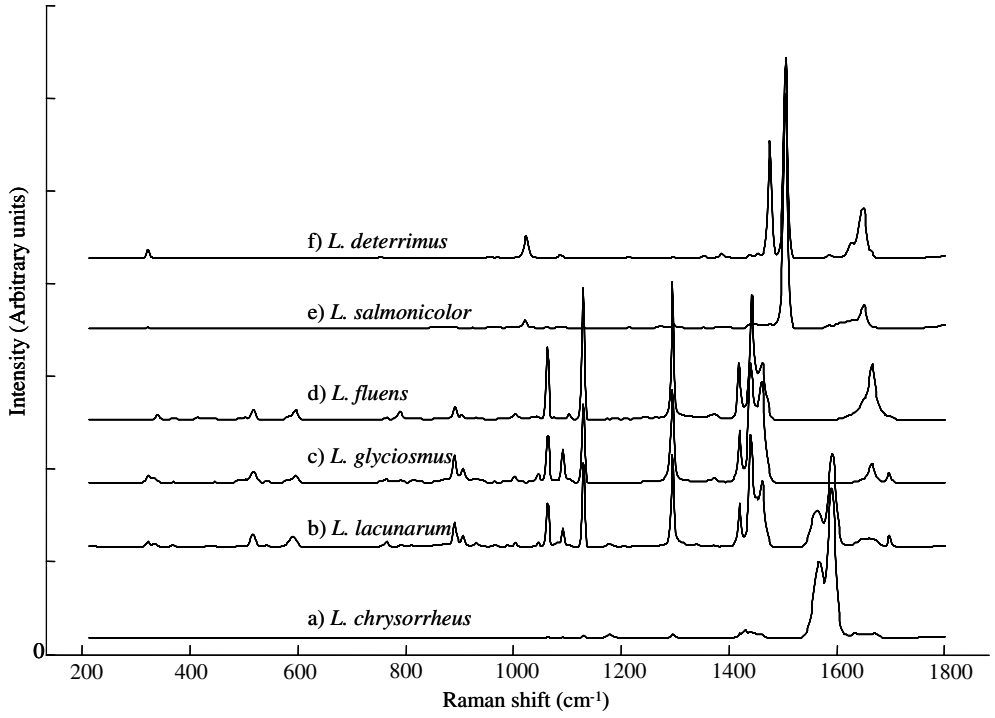

Fig. 6. Symmetry axis signal of the 2D synchronous latex spectra (power spectrum) of different *Lactarius* species.

of its latex (Fig. 6a) are completely different compared to *L. lacunarum*, *L. glyciosmus* and *L. fluens*. Very intense signals are observed at 1567 and 1598 cm^{-1}, whereas no typical Raman bands of aromatic compounds are observed at 543, 590, 1004 and 1046 cm^{-1}. The observed Raman spectra likely result from farnesane sesquiterpenes, which were reported from *L. porninsis* (*L.* section *Zonarii*) (Gamba-Invernizzi et al., 1993).

L. salmonicolor (*L.* section *Deliciosi*) latex (Fig. 6e) is brightly orange-red coloured, which is reflected in the Raman spectra as well. These are dominated by Raman bands at 1505 and 1650 cm^{-1}. The former could be due to carotenoids having a long-chain of (possibly 13) conjugated carbon–carbon double bonds (Withnall et al., 2003). Bergendorff and Sterner (1988) found, however, highly unsaturated and cyclic azulene structures in the latex of *L. deliciosi* and *L. deterrimus* (both *L.* section *Deliciosi*). The latex of *L. salmonicolor* has therefore a totally different composition which is confirmed by a disappearing Raman band at 1022 cm^{-1} and the formation of Raman band at 1274 cm^{-1}.

The latex of *L. deterrimus* (*L.* section *Deliciosi*, Fig. 6f) is bright red coloured and the Raman bands which show the highest time-correlation are observed at 1023, 1476, 1505, 1637 and 1650 cm^{-1}. As the Raman spectra of latexes of *L. deterrimus* and *L. salmonicolor* show similar chemical characters, it can be considered that the sesquiterpenoid precursor has a totally different organic skeleton (azulene skeleton) compared to the other species inspected. The existence of different types of sesquiterpenoid precursors and the linkage to taxonomic groups, was also reported previously in NMR-spectroscopic studies (Hansson et al., 1995).

2.4. Method evaluation

The chemical modifications in *Lactarius* latex may be difficult to reproduce, because they are induced enzymatically. The latex is most efficiently collected by cutting a small part from the pileus of a young specimen. A lot of latex drops can often be collected from the cutting face and were transferred directly to a CaF_2 slide. However, when no precautions were taken and when only latex is transferred, no chemical modification can be observed at all. This is a confirmation of the presence and necessity of enzymes and is of importance when unreacted *Lactarius* latex has to be observed. We therefore found during the experiments that the most critical part was to find a way to transfer reproducible amounts of enzymes and latex to a CaF_2 slide, which will be further explained. Another important factor is that during the experiment, the latex is drying. This may bring the latex out of focus as well as it may prohibit the chemical reactions, since the mobility of reagents lowers the speed of the reactions.

Sterner et al. (1985a) noticed that the chemical reactions in the latex could be induced with silica gel or polar solvents such as acetone or methanol. This was merely a side effect of chromatography, but these polar solvents could eventually be used to study the latex. Moreover in previous studies (Bergendorff and Sterner, 1988; Clericuzio and Sterner, 1997; Daniewski et al., 1996; De Bernardi et al., 1992, 1993; Gamba-Invernizzi et al., 1993; Hansson et al., 1995; Sterner et al., 1985b) latex was extracted using ethanol, ethylacetate, hexane or dichloromethane. In this way, other terpenes which are not present in the latex could be

analysed and detected as well. We preferred not to use solvents in our experiments, as this might lead to unwanted side reactions.

Because solvents could induce side reactions, the natural situation was approximated as much as possible in this research project by adding a small part of a lamella to the drop of latex. Lipases and other enzymes, which are responsible for the chemical modifications, are present in the cell walls of specialised hyphae (Daniewski et al., 1995). The enzymes are therefore shielded and separated from the latex in the latex hyphae. Contact of exuded latex with the bruised fungus body (part of lamella) starts the reactions. Alternatively, the enzymes of the fungus can be transferred by cutting the pileus at the cutting face a few times using a scalpel, while afterwards the scalpel is brought into contact with the latex by dipping it in the latex. Subsequently, the sample was brought into the focus of the laser beam.

Taking possible issues related to the drying process into account, a medium-thick layer of latex was guaranteed. To anticipate focus problems due to latex drying, the autofocus function of the spectrometer in the Holograms software was used. Thus, we were able to analyse *Lactarius* latex in a reproducible way.

3. Conclusion

It can be concluded that Raman spectroscopy is perfectly able to monitor chemical reactions in *Lactarius* latex. By using 2D correlation analysis of the Raman spectra, the reaction patterns in *L. lacunarum* latex are investigated. Four major clusters of functional groups are affected by the chemical reactions, indicating at least four successive chemical reactions. The reaction pathway of *L. lacunarum* latex starts with an enzymatically induced ester splitting reaction forming myristic acid and the bioactive aromatic compound. Raman spectra of *L.* latex of different species are comparable, although notorious spectral differences can be observed. Raman spectroscopy can be thus used to scan the chemical nature of the latex sesquiterpenoids. Therefore, Raman spectroscopy can be regarded as a novel chemical tool for the analysis of *Lactarius* latex.

4. Experimental

4.1. Instrumentation

A Kaiser System Hololab 5000R modular Raman spectrometer (*f*/1.8) was used. Using an excitation wavelength of 785 nm, samples are exposed to laser light with a net power of 35–40 mW at the sample. The laser (Toptica Photonics AG, Martinsried/Munich, Germany) is coupled to the microscope and spectrograph by means of optical fibres (N.A. 15 μm). One minute acquisition time was used for recording all spectra. The system includes a 100× objective lens (PL Fluotar L, 100×/0.75, W.D. 4.7 mm, Leica) to focus the laser light on the sample, a HoloSpec VPT System spectrograph and a back-illuminated deep depletion CCD for detection of the scattered photons. The spectrometer has a resolution of approximately 4 cm^{-1} and the standard deviation on the determination of band positions is approximately 0.3 cm^{-1}.

4.2. Samples and spectral acquisition

Fresh specimens were collected in the field at different locations in Flanders (Belgium). To collect the latex from the specimen, a small part of the pileus was cut out. Drops of the latex were collected on a CaF_2 slide and when the layer was too thick, the latex was smeared out. As the reactions in the latex are enzymatic transformations, a sufficient concentration of enzymes should be present in the latex. This was achieved by adding a small part of a lamella to the drop of latex or by cutting the pileus at the cutting faces a few times using a scalpel and rinsing the scalpel with the latex. Both methods can be practiced and for each specimen the most appropriate method was used. Once the sample was mounted in the spectrometer, 1 min acquisition spectra were collected at different locations to eliminate excessive heating effects and laser-light induced reactions. The following samples were used in this research project:

L. chrysorrheus, Belgium, Dourbes, Tienne aux Pacquis, with *Quercus* and *Fagus*, calcareous soil, IFBL J5.31.43, 19 sep 2004, KDG 2004-26.
L. deterrimus, Belgium, Hautes Fagnes, rés. for. Ruhrbush (Butgenbach/Elsenborn), road side with *Picea*, IFBL G8.25.22, 15 sep 2004, KDG 2004-21.
L. fluens, Belgium, La Reine Pédauque, IFBL H6.28.44, under *Fagus*, 16 oct 2005, KDG 2005-66 (GENT).
L. glyciosmus, Belgium, Fort van Steendorp, IFBL C4.54.11, in grass field with *Betula*, 10 oct 2005, KDG 2005-53.
L. lacunarum, Belgium, Ghent, university campus De Sterre, IFBL D3.22.43, in grass field with *Betula*, 24 oct 2005, KDG 2005-76 (GENT).
L. salmonicolor, Belgium, La Reine Pédauque, IFBL H6.28.44, under *Picea*, 16 oct 2005, KDG 2005-75 (GENT).

All specimens are kept in the herbarium of Ghent University (Herbarium Universitatis Gandavensis, GENT, Belgium).

The fatty acids, stearic acid and myristic acid were obtained as reference products from Sigma–Aldrich and had purity higher than 99%.

4.3. Instrument calibration

The Holograms 4.0 software, which was delivered along with the Raman spectrometer, was used for spectral recording. The spectra were exported to Matlab 6.5 R13

(the MathWorks, Inc.) where the calibration was performed using the method outlined by Hutsebaut et al. (2005). For accurate Raman shift calibration 4-acetamidophenol, acetonitrile/toluene (50/50 v/v), 1,4-bis-(2-methylstyryl)-benzene, cyclohexane, naphthalene, polystyrene and sulphur were used. Spectra were corrected for dark noise and optics. Subsequently, an intensity-calibration has been performed using the known wavelengths of the atomic lines from neon and a well calibrated tungsten bulb of Optronic Laboratories, Inc., Orlando, USA. operating at 6.500 A.

4.4. Data preprocessing

The baseline of the calibrated spectra is subtracted automatically using an iterative polynomial fitting procedure outlined by Lieber and Mahadevan-Jansen (2003). For this background subtraction algorithm, 200 iterations and a fifth-order polynomial were used.

4.5. 2D correlation spectroscopy

2D correlation analysis was performed using in-house written Matlab routines. In order to correct for possible differences in collection intervals of the spectra, 2D correlation analysis for unevenly spaced data, using the Hilbert transform method, was used (Noda and Ozaki, 2004). Prior to 2D correlation analysis, the spectra were mean-centred.

Acknowledgements

The authors greatly acknowledge the financial support of the Research Foundation Flanders (FWO-Vlaanderen). Kris De Gussem and Joke De Gelder are research assistants of the Research Foundation Flanders and they wish to thank this organisation for their Ph.D. grant. Peter Vandenabeele is also thankful to this organisation for his post-doctoral grant.

References

Bergendorff, O., Sterner, O., 1988. The sesquiterpenes of *Lactarius deliciosus* and *Lactarius deterrimus*. Phytochemistry 27, 97–100.

Camazine, S., Lupo, A.T., 1984. Labile toxic compounds of the Lactarii – the role of the laticiferous hyphae as a storage depot for precursors of pungent dialdehydes. Mycologia 76, 355–358.

Carlile, M.J., Watkinson, S.C., 1994. The Fungi. Academic press, London.

Clericuzio, M., Sterner, O., 1997. Conversion of velutinal esters in the fruit bodies of *Russula cuprea*. Phytochemistry 45, 1569–1572.

Daniewski, W.M., Gumulka, M., Przesmycka, D., Ptaszynska, K., Bloszyk, E., Drozdz, B., 1995. Sesquiterpenes of *Lactarius* origin, antifeedant structure–activity relationships. Phytochemistry 38, 1161–1168.

Daniewski, W.M., Gumulka, M., Truszewska, D., Jacobsson, U., Norin, T., 1996. Monohydroxylactones of *Lactarius vellereus*. Phytochemistry 41, 1093–1096.

De Bernardi, M., Vidari, G., Vita-Finzi, P., 1992. The chemistry of *Lactarius fuliginosus* and *Lactarius picinus*. Tetrahedron 48, 7331–7344.

De Bernardi, M., Garlaschelli, L., Toma, L., Vidari, G., Vita-Finzi, P., 1993. The chemical basis of hot-tasting and yellowing of the mushrooms *Lactarius chrysorrheus* and *L. scrobiculatus*. Tetrahedron 49, 1489–1504.

De Gussem, K., Vandenabeele, P., Verbeken, A., Moens, L., 2005. Raman spectroscopic study of *Lactarius* spores (Russulales, Fungi). Spectrochim. Acta A 61, 2896–2908.

Gamba-Invernizzi, A., Garlaschelli, L., Rossi, A., Vidari, G., Vita-Finzi, P., 1993. New farnesane sesquiterpenes from *Lactarius porninsis*. J. Nat. Prod. 56, 1948–1953.

Garlaschelli, L., Mellerio, G., Vidari, G., Vitafinzi, P., 1994. New fatty-acid esters of drimane sesquiterpenes from *Lactarius uvidus*. J. Nat. Prod. 57, 905–910.

Hansson, T., Sterner, O., Strid, Å., 1995. Chemotaxonomic evidence for a division of *Lactarius vellereus* and *L. bertillonii* as different species. Phytochemistry 39, 363–365.

Heilmann-Clausen, J., Verbeken, A., Vesterholt, J., 1998. The Genus *Lactarius* – Fungi of Northern Europe, vol. 2. Svampetryk, Tilst.

Hutsebaut, D., Vandenabeele, P., Moens, L., 2005. Evaluation of an accurate calibration and spectral standardization procedure for Raman spectroscopy. The Analyst 130, 1204–1214.

McCreery, R.L., 2000. Raman spectroscopy for chemical analysis. Chemical Analysis Series, vol. 157. John Wiley & Sons Ltd., New York.

Lieber, C.A., Mahadevan-Jansen, A., 2003. Automated method for subtraction of fluorescence from biological Raman spectra. Appl. Spectrosc. 57, 1363–1367.

Noda, I., Ozaki, Y., 2004. Two-dimensional Correlation Spectroscopy – Applications in Vibrational and Optical Spectroscopy. John Wiley & Sons Ltd., Chichester.

Socrates, G., 2001. Infrared and Raman Characteristic Group Frequencies – Tables and Charts, third ed. John Wiley & Sons Ltd., Chichester.

Sterner, O., Bergman, R., Kihlberg, J., Oluwadiya, J., Wickberg, B., Vidari, G., De Bernardi, M., Demarchi, F., Fronza, G., Finzi, P.V., 1985a. Basidiomycete sesquiterpenes – the silica-gel induced degradation of velutinal derivatives. J. Org. Chem. 50, 950–953.

Sterner, O., Bergman, R., Kihlberg, J., Wickberg, B., 1985b. The sesquiterpenes of *Lactarius vellereus* and their role in a proposed chemical defense system. J. Nat. Prod. 48, 279–288.

Suortti, T., von Wright, A., Koskinen, A., 1983. Necatorin, a highly mutagenic compound from *Lactarius necator*. Phytochemistry 22, 2873–2874.

Withnall, R., Chowdhry, B.Z., Silver, J., Edwards, H.G.M., de Oliveira, L.F.C., 2003. Raman spectra of carotenoids in natural products. Spectrochim. Acta A 59, 2207–2212.