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RESEARCH ARTICLE

Vibrational and structural investigations on adipose tissues

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(30 April 2006)

Two types of adipose tissue are found in mammals, including humans: the white adipose tissue (WAT) and the brown adipose tissue (BAT). The WAT has a major role in lipid storage and body thermal insulation, while the BAT is a thermogenic tissue that produces heat by oxidizing fatty acids. Both structural characterization and spectroscopic discrimination of these different adipose tissues are matter of current interest, also in view of possible medical and bio-technological applications. In this work, vibrational and structural investigations on rat BAT and WAT have carried out by means of infrared and Raman micro-spectroscopy. By comparing their vibrational spectra some characteristic peaks have been identified as useful markers to discriminate between the two tissues. Moreover, a quantitative evaluation of the relative degree of unsaturation of the triglyceride component in WAT and BAT has been derived. These results corroborate the vibrational spectroscopy as a reliable and minimally invasive tool in biological and medical research.

1. INTRODUCTION

In the last decades, medical research on adipose tissues has undergone a remarkable increase of interest recognizing to them not only the role of energy reserve and of structural-mechanical support, but also well-defined biochemical and metabolic disfunctions leading to several diseases, like obesity, heart-disease, diabetes, hypertension and atherosclerosis [1]. The adipose organ consists of two types of tissues, the white adipose tissue (WAT) and the brown adipose tissue (BAT), which basically differ in the degree of vascularization and innervation, with a protein content much higher in the latter tissue than in the former [2, 3]. Both tissues are endowed with the ability to accumulate lipids, essentially triglycerides (TG), although with different purposes: BAT burns lipids to ensure the thermal homeostasis of the body, while WAT stores lipids to provide energy to neighboring organs and tissues, and to assure body thermal insulation. The relative amount of white and brown adipose cells can vary in mammals according to feeding [4, 5], age [6] and environmental temperature [7].

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1 In recent years, vibrational spectroscopy has been extensively employed in bi-
2 ological and medical research, due to its good sensitivity to molecular structure
3 [8]. In particular, Raman spectroscopy provides a suitable approach for fast and
4 non-destructive investigations of adipose tissues and fatty acids, thus allowing to
5 identify the different constituents of the tissue on the basis of their vibrational
6 spectra. For instance, micro-Raman spectroscopy has been exploited either to char-
7 acterize adipose tissues obtained from some animal species [9, 10], or to derive an
8 evaluation unsaturation degree of fatty acids in the adipose tissue coming from
9 different regions of the salmon body [11]. On the other hand, a deeper understand-
10 ing of the composition of adipose tissues requires a finer investigation that can
11 be gained by means of nuclear magnetic resonance (NMR) spectroscopy [12] and
12 nuclear magnetic resonance imaging (MRI) [13, 14].

13 In the following, we report on the results of both vibrational and structural in-
14 vestigations on ex-vivo WAT and BAT samples, obtained by rat biopsy, carried
15 out by micro-Raman and micro-FTIR spectroscopy. After preliminary tests aimed
16 at defining a suitable sample-preparation protocol, Raman and IR spectra of both
17 tissues have been compared in order to identify the vibrational properties of their
18 different components. Furthermore, a quantitative evaluation of the relative unsat-
19 uration degree of fatty acid chains within the two tissues has been derived through
20 the comparative analysis of the Raman intensity of C=C stretching mode of their
21 TG components.

26 2. MATERIALS AND METHODS

27 The experiments were carried out following the NIH Guide for the Use Care of
28 Laboratory Animals and the European Community Council (86/609/EEC) direc-
29 tive. Wistar rats were sacrificed to obtain proper specimens of both WAT and
30 BAT tissues. All rats were housed at constant temperature (20-24 °C) and fed
31 "ad-libitum". Young animal were 16-day-old. Both interscapular and periepidydi-
32 mal lipid tissues were removed from each sacrificed rat as representative samples
33 of WAT and BAT, respectively. Measurements were run on the tissue immediately
34 after its removal, in order to avoid the occurrence of spurious effects caused by
35 the preparation protocol of histological tissues, especially by the formalin fixing
36 [15, 16].

37 Total lipids of the adipose tissue were extracted following a modified Folch
38 method [17] by using a 1:1 (v/v) mixture of chloroform/methanol. Proteins con-
39 taining fraction was obtained as a dry residue after removing the organic soluble
40 part (lipids) of this raw extract. The latter was separated into fractions containing
41 almost pure TG and phospholipids (PL) through flash chromatography (FC) on sil-
42 ica gel (Kieselgel 60, Merck) using a chloroform/methanol gradient elution system.
43 These fractions were passed through a nitrogen stream, and stored at temperature
44 below -20 °C until analyzed.

45 Micro-Raman spectra were excited by the 530.9 nm line of a mixed Ar-Kr
46 ion gas laser and recorded in backscattering geometry at room temperature us-
47 ing a triple-monochromator (Horiba-Jobin Yvon, model T64000), set in double-
48 subtractive/single configuration, and equipped with holographic gratings having
49 1800 lines/mm. The scattered radiation was detected at the spectrograph output
50 by a charge-coupled device (CCD) detector, with 1024 x 256 pixels, cooled by li-
51 quid nitrogen. The laser power on the sample surface was kept below 6.5 ± 0.1 mW,
52 and was focused on a spot of 2 μm through the lens of a objective (100x / NA
53 = 0.9). The spectral resolution was better than 0.6 cm^{-1} /pixel while the spec-
54 tral linearity of the system was calibrated using a 50:50 (v/v) acetonitrile/toluene
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1 mixture and comparing to frequency standards from the American Standard Test-
 2 ing Method ASTM E 1840 (1996). Repeated micro-Raman measurements were
 3 carried out under the same experimental conditions from several regions of the
 4 same sample, obtaining very reproducible spectra for WAT tissue, while in the
 5 BAT case the spectra showed a remarkable dependence on the morphology of the
 6 sampled regions. Also in this case, however, the spectra collected from morpholog-
 7 ically similar regions, especially from those richest in lipid component, showed a
 8 good reproducibility (the mean error of peak intensity was of the order of $\pm 3\%$).
 9 The recorded spectra were processed to remove artifacts due to cosmic rays, while
 10 the luminescence background was removed by comparing replicate spectra of each
 11 sample over the whole spectral range.
 12

13 For micro FTIR measurements 7 μm -thick slices of tissue were deposited on 1
 14 mm thick BaF_2 supports and were imaged using a Bruker Vertex 70 spectrometer
 15 coupled to a Hyperion 3000 vis/IR microscope equipped with a single HgCdTe
 16 photoconductive MCT detector and a 15x / NA = 0.4 objective. FTIR spectra
 17 were collected in transmission mode at 4 cm^{-1} resolution on a $50 \times 50\ \mu\text{m}^2$ area
 18 by co-adding 64 scans. Absorbance spectra were baseline corrected in the whole
 19 range with a concave rubberband behaviour using the Bruker OPUS 5.5 software.
 20 Repeated micro-FTIR measurements showed good reproducibility on both adipose
 21 tissues.
 22
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 24

25 3. RESULTS AND DISCUSSION

26
 27 Typical micro-Raman spectra recorded on WAT (a) and BAT (b) in the wavenum-
 28 ber range $800 \div 3300\text{ cm}^{-1}$ are reported in Figure 1. The spectra are normalized
 29 to the intensity of the 2850 cm^{-1} peak, which is the most relevant in both tissues.
 30 Both WAT and BAT Raman spectra are dominated by very intense bands in the
 31
 32

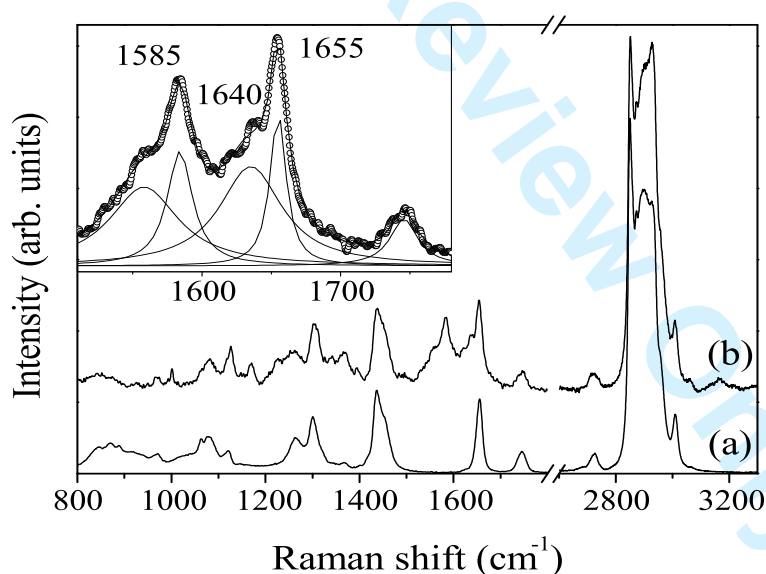


Figure 1. Raman spectra of WAT (a) and BAT (b) in the energy range $800 \div 3300\text{ cm}^{-1}$. Spectra are normalized to the peak intensity at 2850 cm^{-1} . Inset: curve spectrum (b) in the restricted spectral range $1500 \div 1800\text{ cm}^{-1}$; experimental data (empty circle) and Lorentzian fit of the data (full line). Spectrum (b) is vertically shifted for the sake of clarity.

region $2800 \div 3100\text{ cm}^{-1}$. These bands are known to be due either to symmetric or to a-symmetric stretching vibrations of the $-\text{CH}_2-$ and $-\text{CH}_3-$ groups, mostly belonging to the TG component.

Another remarkable spectral feature, observed in both tissues, is the peak at 1655 cm^{-1} . This peak can be attributed to the symmetric stretching mode of the C=C bonds of triglyceride component[18]. Besides, several other common Raman bands are observed in the scanned spectral range. Despite their overall similarity, the spectra of Figure 1 show significant differences, mainly concerning vibration modes involving the amidic groups of proteins. So, for instance, a quite-strong peak at 1585 cm^{-1} is observed only in the spectrum of BAT (fig. 1, inset). This peak can be properly used as reliable signature of brown adipose tissue. Raman spectrum of BAT shows also another spectral component centred at about 1640 cm^{-1} (also see fig. 1, inset), which can be assigned to the amide I band in proteins ($1640 \div 1650\text{ cm}^{-1}$)[19].

Strong support to these assignments is given by FTIR measurements on WAT and BAT. Their absorbance spectra are reported in figure 2 (a) and (b), respectively. Two intense bands at 1545 and 1650 cm^{-1} , assigned to amide II and amide I

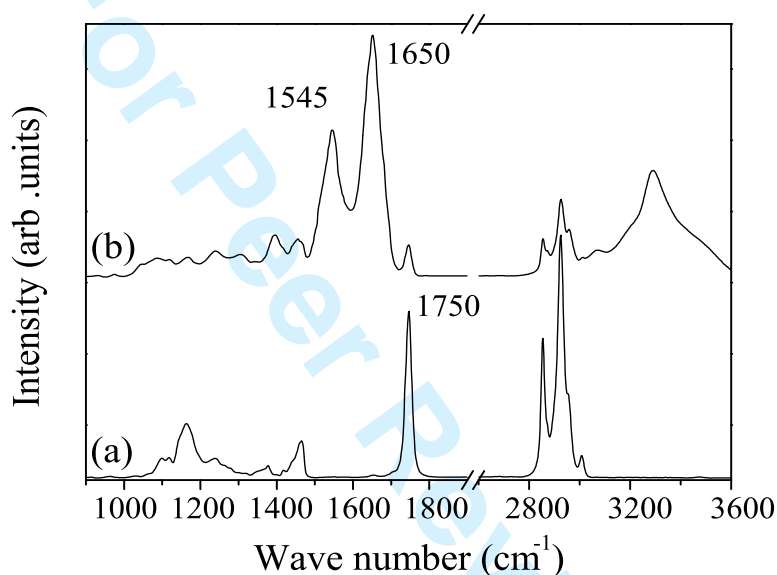


Figure 2. Spectra of WAT (a) and BAT (b) acquired through FTIR micro-spectroscopy of *ex-vivo* tissue. The spectra are normalized to the maximum peak height for the sake of clarity.

vibrations [7], respectively, are clearly seen in the IR spectrum of BAT, where a small peak centred at 1750 cm^{-1} is also observed. In contrast, in the IR spectrum of WAT (Figure 2 (a)) the peak at 1750 cm^{-1} , due to C=O stretching in TG, turns out to be one of the main spectral features, while the bands assigned to the amide I and II disappear. Therefore, in the IR spectra of BAT the above-cited frequencies at 1545 and 1650 cm^{-1} can be chosen as reliable tissue-markers.

On the basis of the previous considerations, the composition of the brown adipose tissue results more complex than that of the white one. Therefore, for a finer spectral analysis of the BAT components, TG, PL and protein residue were extracted from this tissue and separately measured by micro-Raman spectroscopy. The corresponding spectra, observed in the spectral range $800 \div 3300\text{ cm}^{-1}$, are shown in Figure 3, arbitrarily normalized for the sake of clarity. As a whole, Raman spectrum of the TG component (Figure 3 (c)) is very similar to the Raman spectrum of WAT (Figure 1 (a)), confirming that TG constitute indeed the main component of the white adipose tissue. On the other hand, the presence of a protein component in BAT can be clearly inferred by comparing the Raman spectrum Figure 3 (a) with the spectrum Figure 1 (b). Raman features of the most abundant triglyceride component, present in both adipose tissues, allow to get further

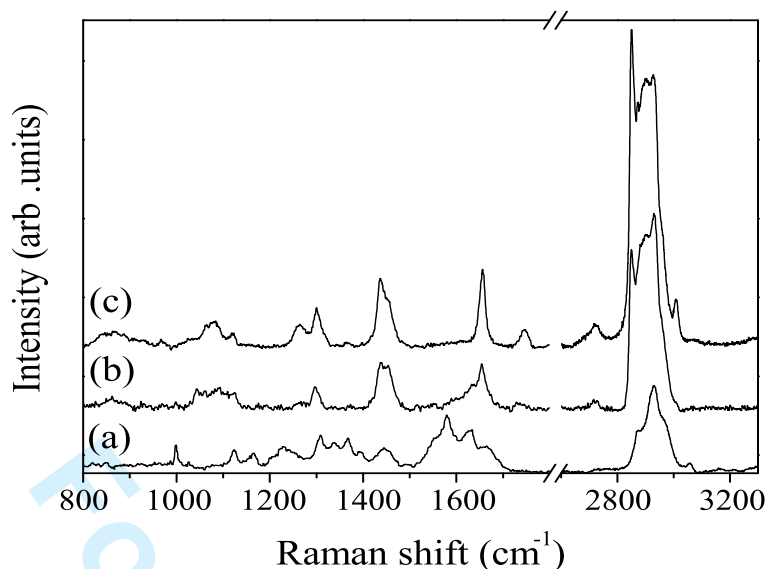


Figure 3. Raman spectra of the separated components of BAT: protein residue (a), PL (b) and TG (c).

insight on the chemical differences between WAT and BAT. In fact, an important functional role is played in adipose tissues by the degree of unsaturation, which is related to the number of double C=C bonds present in the triglyceride chains. Obviously, also the spectral intensity of C=C bands in TG is expected to depend on such a number.

Raman spectra of the triglyceride component, extracted from a sample of white (open circles) and brown (solid line) adipose tissue are reported in Figure 4. Here, the baseline corrected spectra have been normalized to the area of the peak centred at 1745 cm^{-1} , which is attributed to the C=O groups stretching vibrations [18]. This normalization procedure was adopted to take into account the chemi-

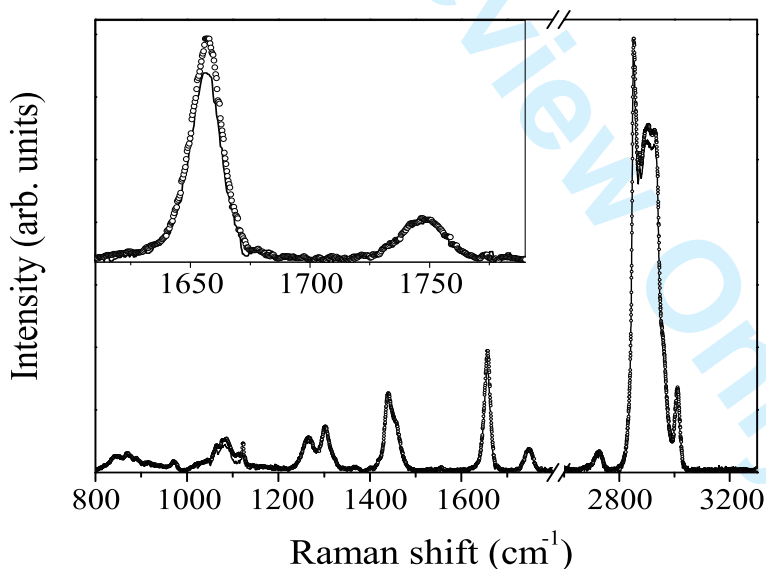


Figure 4. Raman spectra of TG component extracted from WAT (open circles) and BAT (solid line) normalized to the integrated intensity of the 1745 cm^{-1} peak. Inset: the same in the restricted energy range $1600 \div 1800\text{ cm}^{-1}$.

cal structure of TG, where the number of the C=O groups is expected to be the same in both tissues, i.e. three carbonyl groups for each TG, independently from

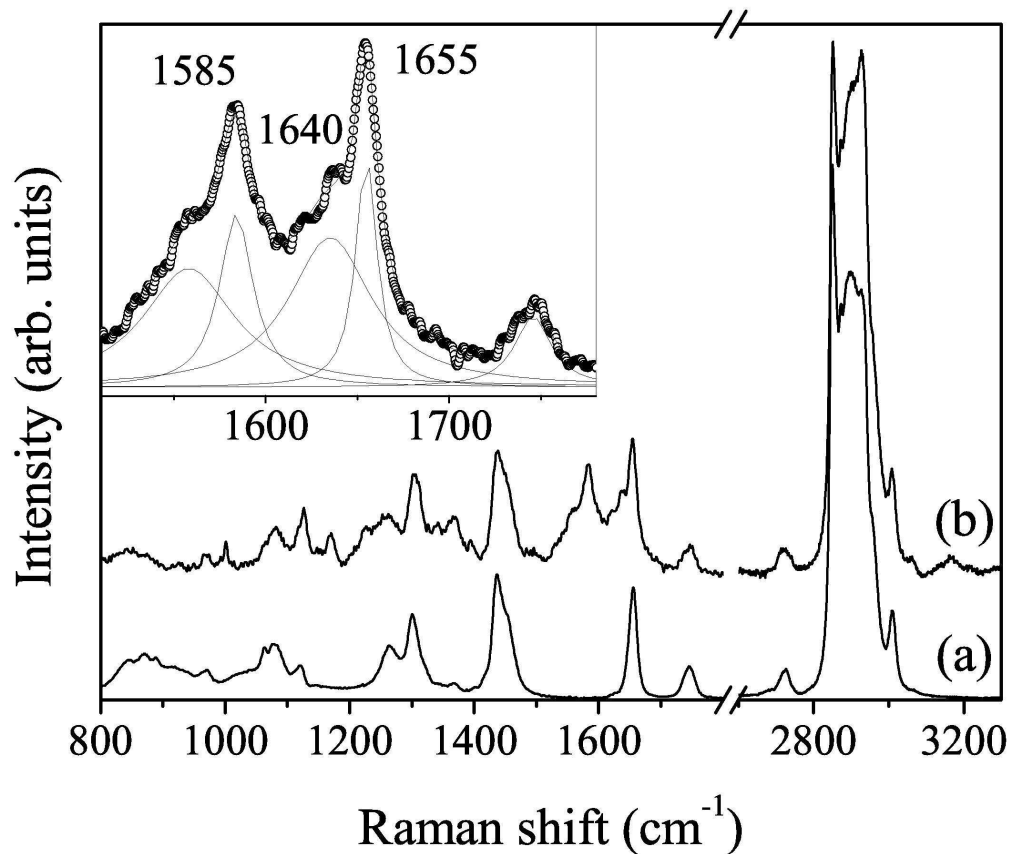
1 the degree of unsaturation of their hydrocarbon chains. In this way, a quantitative
2 evaluation of the relative unsaturation index of TG chains present in both tissues
3 can be derived by measuring the area of the 1655 cm^{-1} peak. By looking at the
4 inset of Figure 4, a small but significant difference between the 1655 cm^{-1} peak in-
5 tensities of WAT (open circles) and BAT (solid line) is clearly observable. The ratio
6 between the intensities of this peak, evaluated for the two tissues, corresponds to
7 the BAT/WAT unsaturation index, and turns out equal to 0.86 ± 0.05 . This result
8 is in substantial agreement with the unsaturation index obtained from $^1\text{H-NMR}$
9 spectra of raw extracts of BAT and WAT measures (0.87 ± 0.05) as evaluated
10 following the methodology described in [12].
11
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13 4. CONCLUSIONS

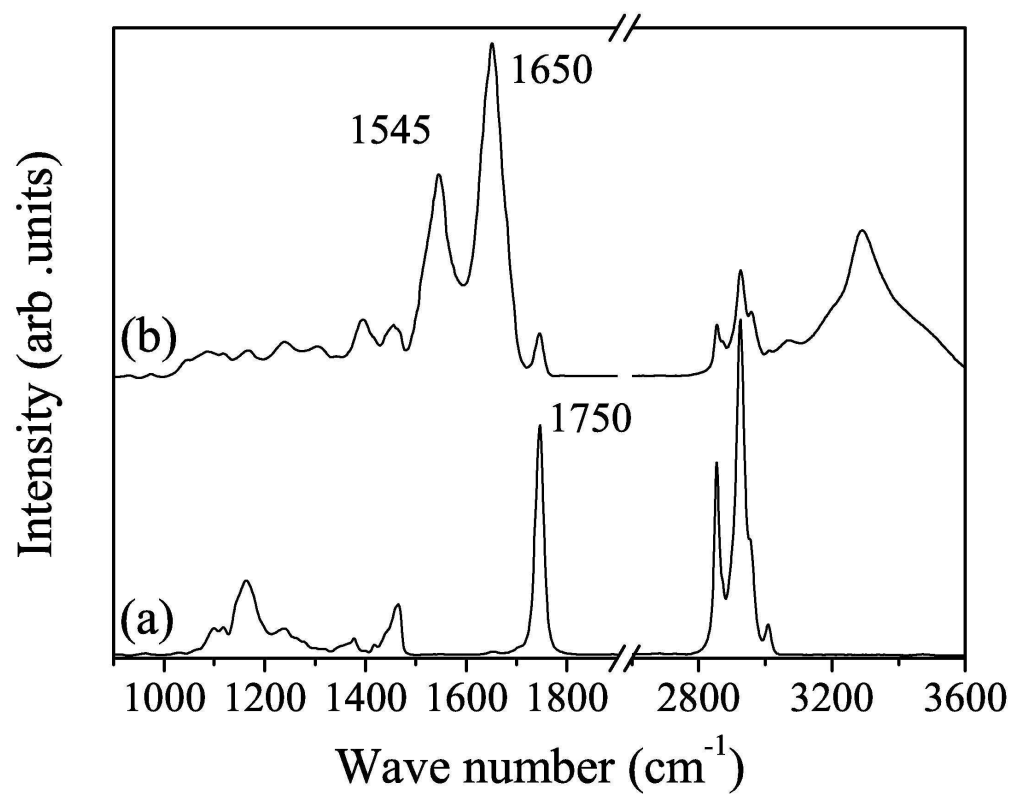
14
15 The vibrational properties of different adipose tissues obtained from rat biopsies,
16 WAT and BAT, have been investigated by means of Raman and FTIR micro-
17 spectroscopy. By an accurate comparative analysis of the spectra acquired on *ex-*
18 *vivo* tissue, some characteristic vibrational peaks have been identified as useful
19 markers able to discriminate between different adipose tissues. In particular, the
20 Raman spectra of the three main components of BAT (TG, PL and proteins) allow
21 to assign the main spectral features observed in BAT. Finally, the relative degree of
22 unsaturation of the tryglyceride component in WAT and BAT has been evaluated
23 and our Raman estimation turns out in good agreement with the $^1\text{H} - \text{NMR}$
24 results.
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30 References

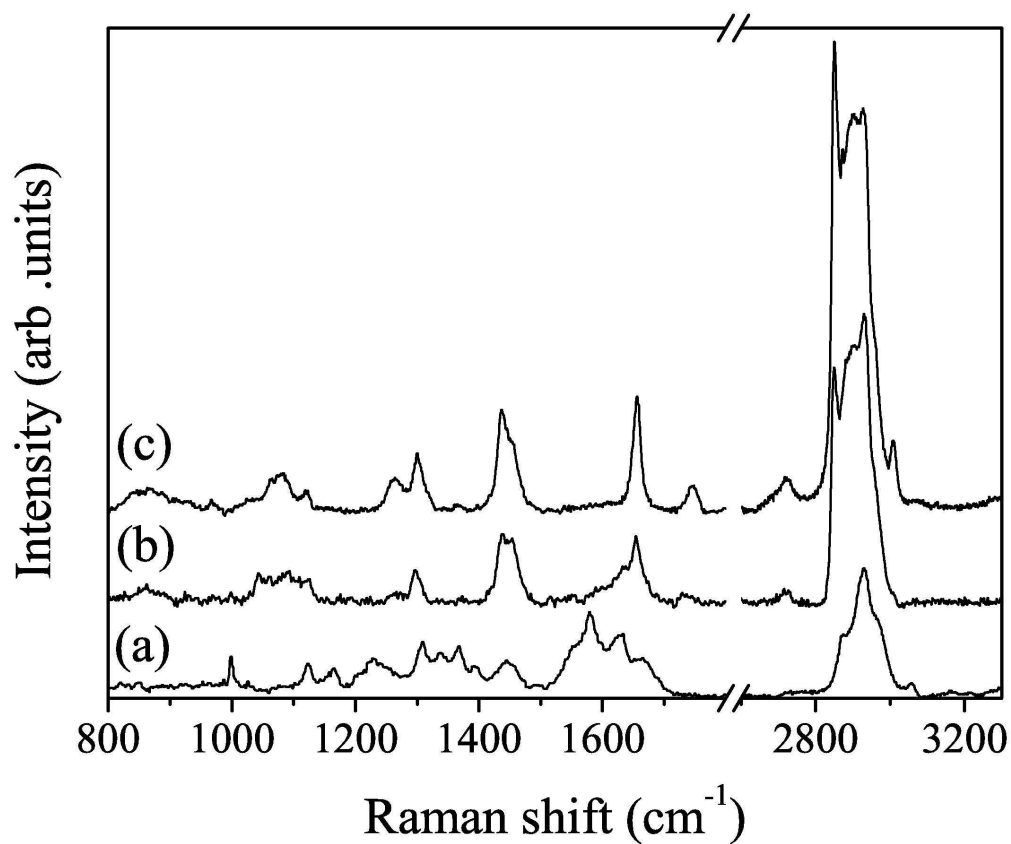
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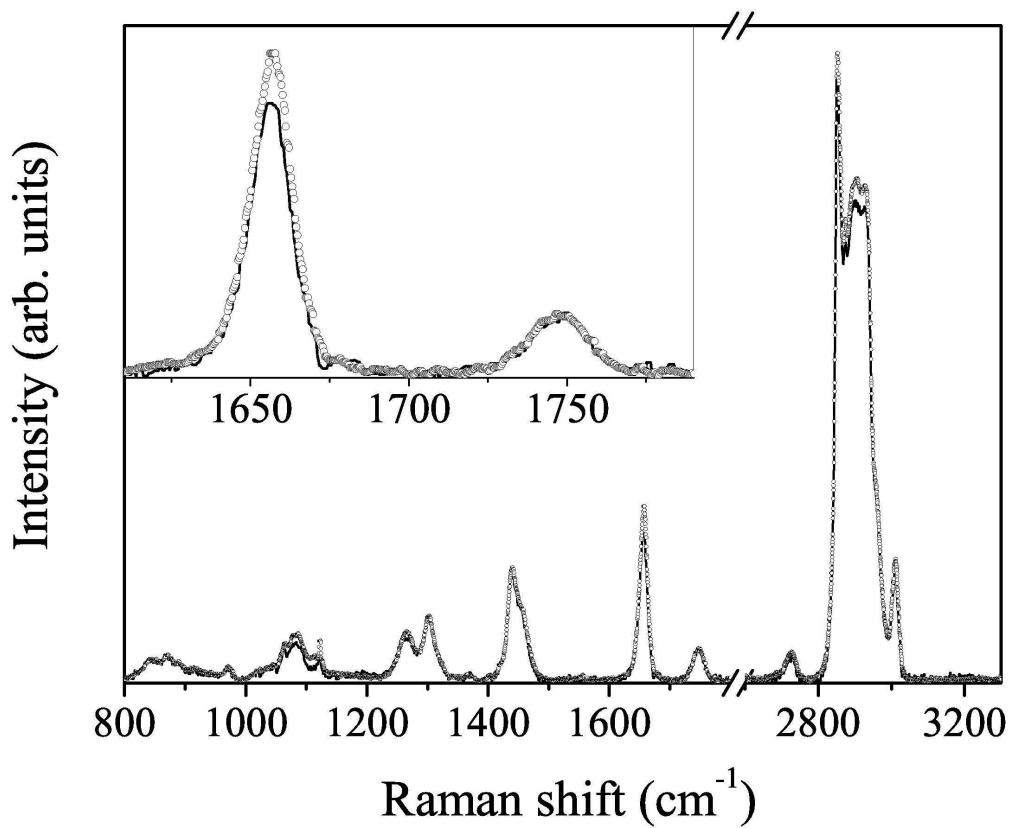
Raman spectra of WAT (a) and BAT (b) in the energy range 800-3300 cm^{-1} . Spectra are normalized to the peak intensity at 2850 cm^{-1} . Inset: curve spectrum (b) in the restricted spectral range 1500-1800 cm^{-1} ; experimental data (empty circle) and Lorentzian fit of the data (full line). Spectrum (b) is vertically shifted for the sake of clarity.
88x75mm (600 x 600 DPI)



Spectra of WAT (a) and BAT (b) acquired through FTIR micro-spectroscopy of ex-vivo tissue. The spectra are normalized to the maximum peak height for the sake of clarity.
91x71mm (600 x 600 DPI)



36 Raman spectra of the separated components of BAT: protein residue (a), PL (b) and TG (c).
37 89x75mm (600 x 600 DPI)



Raman spectra of TG component extracted from WAT (open circles) and BAT (solid line) normalized to the integrated intensity of the 1745 cm^{-1} peak. Inset: the same in the restricted energy range 1600-1800 cm^{-1} .
91x75mm (600 x 600 DPI)