



Raman spectroscopy characterization of the major classes of plasma lipoproteins

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ABSTRACT

Raman spectroscopy has been vastly employed for the characterization of different bio-molecular species spanning from single protein to the in-vivo analysis of tissues. However, despite the huge work done, a detailed description of the Raman spectra acquired from the main classes of plasma lipoproteins is still missing. In this work, we extracted the major classes of lipoproteins: the triacylglycerol-rich very low density lipoproteins (VLDL); and the more cholesterol-rich low density lipoproteins (LDL) and high density lipoproteins (HDL); from human plasma of six fasting healthy volunteers. The extracted lipoproteins were dried on CaF₂ slides and analysed using a 633 nm laser line non-in resonance with the carotenoids present in the sample. The obtained spectra showed peaks relative to the different biomolecules composing lipoproteins: cholesterol, triglycerides, membrane lipids, carotenoids, and apolipoproteins (proteins). The intensity of the peaks from lipids and proteins is well in accordance with the measured composition of lipoproteins; but the information is acquired in a much faster way by Raman spectroscopy. Besides, Raman spectroscopy provides easily information on the levels of carotenoids and unsaturated fatty acid present in the samples. Overall, our data provide a clear comprehension of the Raman spectra from lipoproteins and suggest that Raman spectroscopy could be a viable approach for the fast characterization of lipoproteins.

1. Introduction

Raman spectroscopy (RS) has been extensively used for the characterization of many biochemical species and extensive literature is now available proving that RS can be used for the characterization of cells, tissues, and biofluids [1–3]. More recently, the coupling of RS with multivariate statistical analysis was successfully applied for the analysis and classification of patients affected by several diseases [4–6]. Until now, most of the studies seem to focus on the analysis of proteins and other small metabolites (such as carotenoids) present in the samples, while the analysis of biological lipids by RS remained a slightly less explored field of study, even if they have very clear spectra [7,8].

Due to their hydrophobic nature, lipids travel in blood inside lipoproteins (LPs); complex particles responsible for the transport of fatty acids, cholesterol, carotenes, and vitamin E. LPs fractionation and quantification are a matter of primary interest in clinical medicine since several large-scale epidemiological studies showed that elevated concentrations of fasting cholesterol and triacylglycerols, stored in specific LPs, are associated with an increased incidence of cardiovascular events [9,10]. Therefore, the quantification of cholesterol and triglycerides transported within LPs is a standard clinical practice commonly applied to subjects at risk of cardiovascular events and to those under-

going cardiac rehabilitation [11]. More recently, new researches suggest that LPs are likely related with neurological diseases, such as Alzheimer's disease and Amyotrophic Lateral Sclerosis [12,13].

In the blood of a fasting subject, three major lipoprotein classes are present: the triacylglycerol-rich very low density lipoproteins (VLDL); and the more cholesterol-rich low density lipoproteins (LDL) and high density lipoproteins (HDL).

The main aim of the present work is to demonstrate the suitability of RS as a valuable tool to extract, easily and with a reduced sample preparation, valuable information about the lipid content of the main classes of LPs present in blood: VLDL, LDL, and HDL.

Although RS was previously used to characterize LPs used in functional studies on macrophages, [14] most of the work published until now was focused on the attempt to extract by RS information on LPs content in serum or plasma [15,16]. To the best of our knowledge, a systematic description of the Raman spectra of the main classes of LPs extracted from human plasma of the same subjects is still lacking.

On the contrary, RS is a well-suited tool for this task. In fact, RS was previously used for the characterization of edible oils, such as olive oil or sunflower oil, [17] and as a rapid method to unveil oil adulterations [18]. Equally important, most modern Raman instruments are coupled with microscopy (micro-Raman) and this makes them suitable for

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the analysis of very small amount of samples such as the volumes of LPs extracted from of blood plasma.

In this work, we extracted VLDL, LDL, and HDL by ultracentrifugation from the plasma sample of six healthy volunteers in fasting conditions. For all the subjects, Raman spectra from lipoproteins were collected in the regions between 400 cm^{-1} and 1800 cm^{-1} (low frequency region) and between 2600 and 3200 cm^{-1} (high frequency region). The data here presented show the main bands that are observed on the different LPs and how the intensity ratio of the peaks reflect their different biochemical composition.

2. Material and methods

2.1. Subjects and methods

Six healthy volunteers were recruited for the study. The study participants comprised three women and three men, age 30 ± 3 years. Blood was collected from fasting subjects (at least 12 h after food intake). Blood samples were obtained by venepuncture and were collected in EDTA-containing Vacutainer tubes. Plasma was prepared by centrifugation at 3000 rpm for 20 min at 4 °C, then centrifuged again under the same conditions to completely remove the erythrocytes. Plasma samples were aliquot and stored at -80 °C until used. Subjects gave their written consent to the study.

2.2. Lipoprotein isolation and characterization

Major lipoproteins classes (VLDL, LDL, and HDL) were isolated from plasma by ultracentrifugation in discontinuous KBr density gradient adapting procedure 16 in ref. [19] to "Optima Max" tabletop ultracentrifuge (Beckman Coulter) as previously described [20]. To remove albumin completely, the HDL fraction (density, 1.063–1.210 g/mL) was subjected to a second centrifugation [procedure 15 in ref. [19]. After separation, lipoproteins were dialyzed overnight in 10 mM phosphate buffer pH 7.4 containing 154 mM NaCl. The concentration of proteins was determined by the Lowry method using bovine serum albumin as standard. Lipids were extracted by the Folch procedure [21]. The levels of cholesterol, phospholipid, and triacylglycerols in lipid extracts were determined as previously described [22]. Ultrastructural characterization of VLDL, LDL and HDL has been performed by Transmission Electron microscopy, as follows: a drop of VLDL, LDL or LDL suspension was dried on the Formvar net at RT, stained with uranyl-acetate 1% for 30 s at RT and dried overnight at RT. Samples were evaluated by Transmission Electron Microscopy (Tecnai Spirit, FEI).

2.3. Raman spectra acquisition and analysis

Raman spectra were acquired using an InVia Reflex confocal Raman microscope (Renishaw plc, Wotton-under-Edge, UK) equipped with a He-Ne laser light source operating at 633 nm. The Raman spectrometer was calibrated daily using the band at 520.7 cm^{-1} of a silicon wafer.

In a typical experiment, a 3.5 μL drop of LPs was dropped on the surface of Raman-compatible CaF_2 discs (Crystran, UK) and dried for 20 min at room temperature. The Raman study was performed using a 633 nm excitation laser with 100 % power (around 125 mW at source), a 1200 L/mm grating and a 100x objective (Leica). The diameter of the laser spot on the surface was ≈ 1 μm . Spectra were acquired independently in the region between 400 and 1800 cm^{-1} (low frequency region) and between 2600 and 3200 cm^{-1} (high frequency region) as the sum of five acquisitions of 30 s. Spectra resolution was about 1.1 cm^{-1} . For each sample, five different spectra were collected on different positions of the drop. The software package WIRE 5 (Renishaw, UK) was used for the spectral acquisition and to remove cosmic rays. Background fluorescence was removed by Asymmetric Least

Square Smoothing Baseline using OriginPro software. The spectra acquired for each sample were normalized dividing each value for the intensity of the peak at 1439 cm^{-1} in the low frequency region. For the intensity of the peak at 2894 cm^{-1} in the high frequency region. The average of the five spectra was considered as the final spectrum representative of each subject. Baseline subtraction, spectrum normalization and data analysis were performed using OriginPro, Version 2019 (OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. Isolation of lipoprotein

The chemical composition of LPs extracted by ultracentrifugation is reported in Table 1. Each lipoprotein class showed its expected distinctive chemical composition [23]. VLDL contained approx. 11 ± 1 % protein and 89 ± 5 % total lipids of which cholesterol, triacylglycerol and phospholipid concentrations were approx. 21.7 ± 0.6 %, 49.5 ± 4.2 % and 17.9 ± 0.1 %, respectively. LDL contained approx. 32 ± 2 % protein and 68 ± 4 % total lipids of which cholesterol, triacylglycerol and phospholipid concentrations were approx. 37.5 ± 0.8 %, 6.7 ± 0.5 % and 23.8 ± 0.2 %, respectively. Finally, HDL contained approx. 49 ± 2 % protein and 51 ± 2 % total lipids of which cholesterol, triacylglycerol and phospholipid concentrations were approx. 20.2 ± 0.7 %, 4.5 ± 0.4 % and 26.4 ± 0.1 %, respectively. Extracted LPs were also characterized by Transmission electron microscopy to confirm, through the morphological analysis, the nature of LPs collected in each fraction. As expected, VLDL, LDL, and HDL images reported in Fig. 1 resumes typical features of different classes of LPs.

3.2. Raman Spectra in the high frequency region

As first step, we acquired spectra from LPs in the high frequency region between 2600 and 3200 cm^{-1} . This region of the spectrum is dominated by three main peaks related to of CH_2 bonds at 2851, 2894 and 2930 cm^{-1} in all the samples analysed. Besides, the fourth peak at 3012 cm^{-1} refers to the $=\text{CH}-$ moieties present in the samples. Interestingly, while the position of the peak was constant in VLDL, LDL, and HDL their relative intensity was strongly dependent on the analysed class (Fig. 2 A–C). In particular, we observed a drastic variation of the intensity of the peaks at 2851 cm^{-1} , which mostly refers to CH_2 in lipids, and at 2930 cm^{-1} , that refers to CH_2 in proteins [24]. As expected, the peak relative to lipids decreased its intensity from VLDL to LDL and HDL, while the one relative to the protein content had an opposite behaviour. Notably, the ratio between the two peaks, when calculated for each class of LPs on all the six subjects included, was univocally able to discriminate between the three major classes of LPs (Fig. 2 D). However, because of the limited number of subjects included in the study, these results need to be validated in a larger study. On the contrary, the peak at 3012 cm^{-1} remained constant in both its Raman shift and intensity, regardless of the subject and of the class of LPs analysed.

Table 1
Lipoprotein chemical composition. Data are expressed as mean \pm standard deviation.

% Dry weight	VLDL	LDL	HDL
Protein	10.8 ± 1	31.8 ± 2	48.9 ± 2
Lipids:			
Total	89.1 ± 5	68.2 ± 4	51.1 ± 2
Cholesterol	21.7 ± 0.6	37.5 ± 0.8	20.2 ± 0.7
Triacylglycerols	49.5 ± 4.2	6.7 ± 0.5	4.5 ± 0.4
Phospholipids	17.9 ± 0.1	23.8 ± 0.2	26.4 ± 0.1

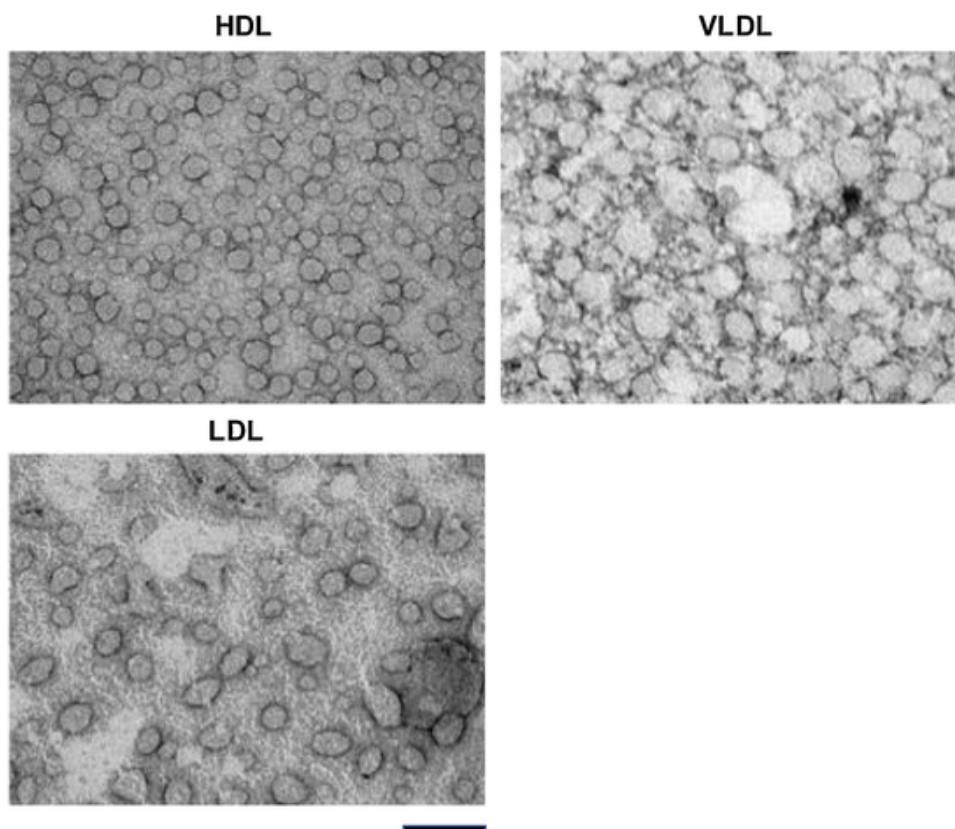


Fig. 1. Transmission electron microscopy images of VLDL, HDL and LDL extracted from a single subject. Scale bar = 100 nm.

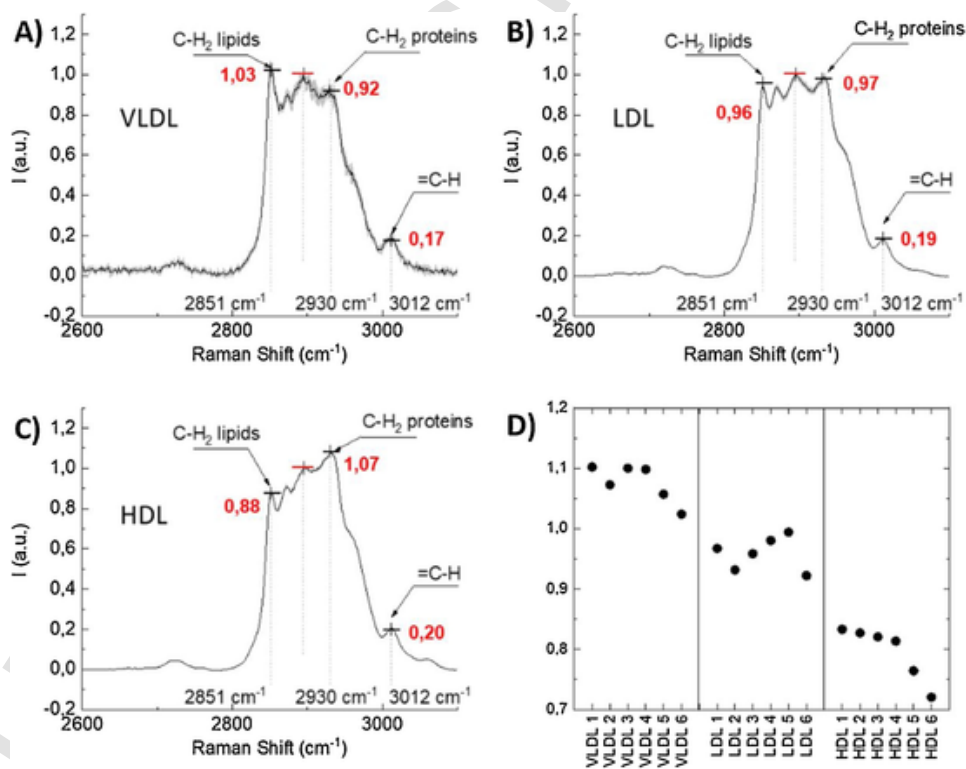


Fig. 2. Raman spectra acquired in the high frequency region on VLDL (A), LDL (B), and HDL (C) extracted from a single subject. The black line is the mean value of the five spectra acquired from each sample. The grey shadow represents the standard deviation of the five measures. D) Ratio between the peaks at 2851 and 2930 cm^{-1} calculated on VLDL, LDL and HDL of the six subjects included in the study.

3.3. Raman spectra in the low frequency region

A second series of Raman spectra were acquired in the low frequency region between 400 and 1800 cm^{-1} . For all the classes of LPs, we were able to acquire very clear spectra (Fig. 3) presenting several peaks clearly related with the major classes of biomolecules present: cholesterol, lipids, protein and antioxidants (carotenoids). Even in this spectra the shift of the peaks was the same in all the different classes of LPs reflecting the fact that their basic constituents remained constant. This region of the spectrum allowed us to clearly evidence the presence of cholesterol, presumably in its esterified form as part of the hydrophobic core of the LPs, as peaks at 700 and 740 cm^{-1} and as a shoulder at 1668 cm^{-1} , [25]. Other peaks at 1061, 1127, 1300 and 1439 cm^{-1} were clearly related with the hydrophobic chains of lipids. Other important peaks that were present in all the spectra, and that re-

ferred to lipids, were the one at 716 cm^{-1} , very characteristic for the asymmetric stretching of choline $\text{N}^+(\text{CH}_3)_3$ groups present in the hydrophilic head of phosphatidylcholine, and the one at 1656 cm^{-1} of trans $\text{CC}=\text{C}$ groups [26]. The first peak, can be used as an indicator of the ratio between the lipids in the hydrophobic core and the ones in the hydrophilic membrane; the latter could represent a way to measure the unsaturated lipids within LPs. The last peak typical of lipid was observed in the region between 1730 and 1745 cm^{-1} due to the $\text{CO}=\text{C}$ stretching bands of the ester bonds [25].

The peaks at 1154 and 1520 cm^{-1} were well known to be peaks relative to the presence of carotenoids and, in general, of antioxidants micronutrients [27]. Other, less intense peaks were typical of aromatic amino acids: 760 and 1355 cm^{-1} for tryptophan; 1003 and 1603 cm^{-1} for phenylalanine [28].

Even in the low frequency region, the intensity of the peaks observed changed between the different classes of LPs and, in some cases, even between the single subjects included in the study. In order to better understand the extent of the variations, we selected three portions of the spectra, particularly relevant and informative, that were analysed more in detail: the region between 650 and 800 cm^{-1} ; the region between 1200 and 1400 cm^{-1} and the region between 1400 and 1800 cm^{-1} .

3.4. Region between 650 and 800 cm^{-1}

The region of the spectra between 650 and 800 cm^{-1} was characterized by the presence of three main peaks at 700, 717 and 740 cm^{-1} . As previously introduced however the intensity of these peaks was not the same in all the classes of LPs studied. In particular the peak of the in-plane deformations of the B ring of cholesterol at 700 cm^{-1} was more intense in LDL spectra (Fig. 4B,D), than in HDL (Fig. 4C,D) and VLDL (Fig. 4A,D). This trend is in good accordance with the composition of LPs measured and with the composition of LPs reported in literature. In fact, LDL are the main vector for the transport of cholesterol and are composed of 35–40 % of their weight by esterified cholesterol and by free cholesterol (see Table 1). On the contrary, the peak at 717 cm^{-1} of the polar head of phosphatidylcholine were more pronounced in HDL than in VLDL and LDL, (Fig. 4E). Even these data were in

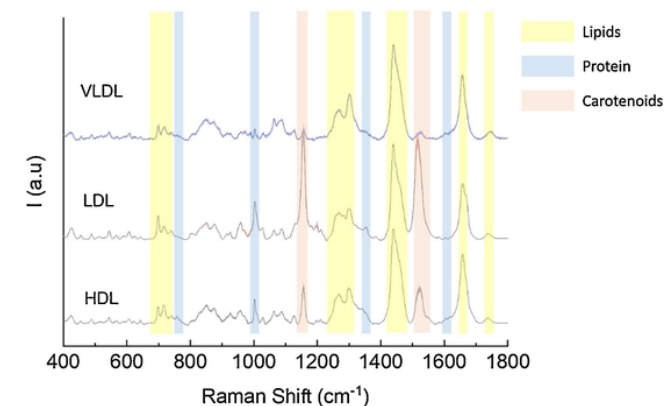


Fig. 3. Raman spectra acquired in the low frequency region on VLDL (top), LDL (middle) and HDL (bottom) extracted from a single subject. The black line is the mean value of the five spectra acquired from each sample. The grey shadow represents the standard deviation of the five measures. Color bands highlight the classes of a biomolecule that are represented by the peaks in the spectra. Yellow bands highlight peaks relative to the lipid content; light blue bands highlight peaks relative to proteins, while the pink band highlight peaks relative to carotenoids.

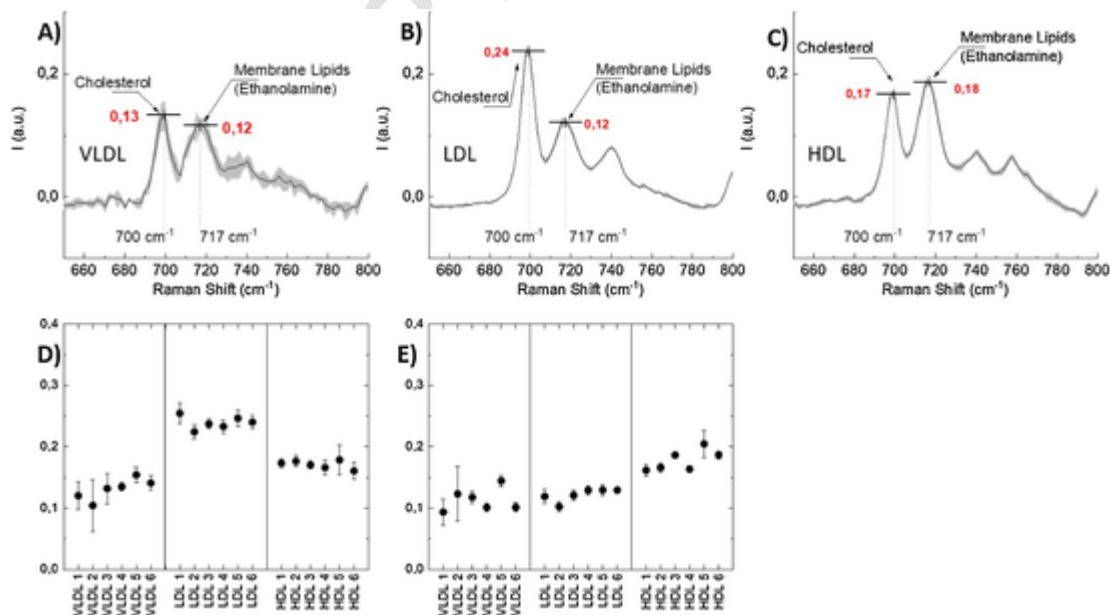


Fig. 4. Raman spectra acquired between 650 and 800 cm^{-1} on VLDL (A), LDL (B) and HDL (C) extracted from a single subject. The black line is the mean value of the five spectra acquired from each sample. The grey shadow represents the standard deviation of the five measures. D) Intensity of the peak at 700 cm^{-1} measured on VLDL, LDL and HDL of the six subjects included in the study. E) Intensity of the peak at 717 cm^{-1} measured on VLDL, LDL and HDL of the six subjects included in the study. In Fig. 4D-E black dots are the mean of the five spectra acquired on each sample, brackets are the standard deviations.

close accordance with the literature. HDL are very small particles (5–20 nm) and, in fasting subjects, their main constituents are the membrane lipids (as phosphatidylcholine) and apolipoproteins. The percentage of membrane lipids is higher in HDL than in other LPs as also appears in Table 1. The good accordance of RS with the composition of LPs was also proven by the presence of the peak of tryptophan at 760 cm^{-1} in HDL spectra where proteins are much more concentrated (about 50 % of dry weight according to our measure). The same peak was almost undetectable in LDL and VLDL, where proteins are significantly diluted.

By comparing the intensity of the two peaks at 700 and 717 cm^{-1} in the different subjects, it was also remarkable to observe how the val-

ues remain rather constant and did not have a large inter-subjects variability.

3.5. Region between 1200 and 1400 cm^{-1}

The region of the spectra between 1200 and 1400 cm^{-1} was characterized by the presence of three main peaks at 1270 , 1300 and 1355 cm^{-1} (Fig. 5A–C). The peak at 1270 cm^{-1} of $=\text{C-H}$ in-plane deformation was very constant in all samples studied and did not provide any significant information to the analysis except to the presence of unsaturated lipids. The peak at 1300 cm^{-1} of CH_2 twisting mode is a marker of the presence of the hydrophobic chains of fatty acids [25].

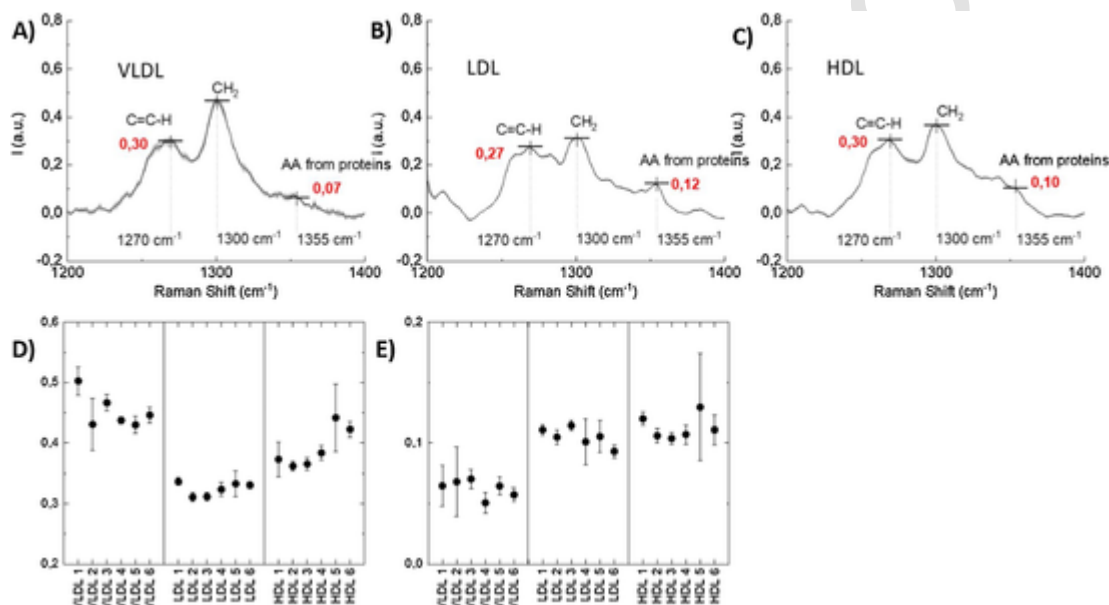


Fig. 5. Raman spectra acquired between 1200 and 1400 cm^{-1} on VLDL (A), LDL (B) and HDL (C) extracted from a single subject. The black line is the mean value of the five spectra acquired from each sample. The grey shadow represents the standard deviation of the five measure. D) Intensity of the peak at 1300 cm^{-1} measured on VLDL, LDL and HDL of the six subjects included in the study. E) Intensity of the peak at 1355 cm^{-1} measured on VLDL, LDL and HDL of the six subjects included in the study. In Fig. 4D-E black dots are the mean of the five spectra acquired on each sample, brackets are the standard deviations.

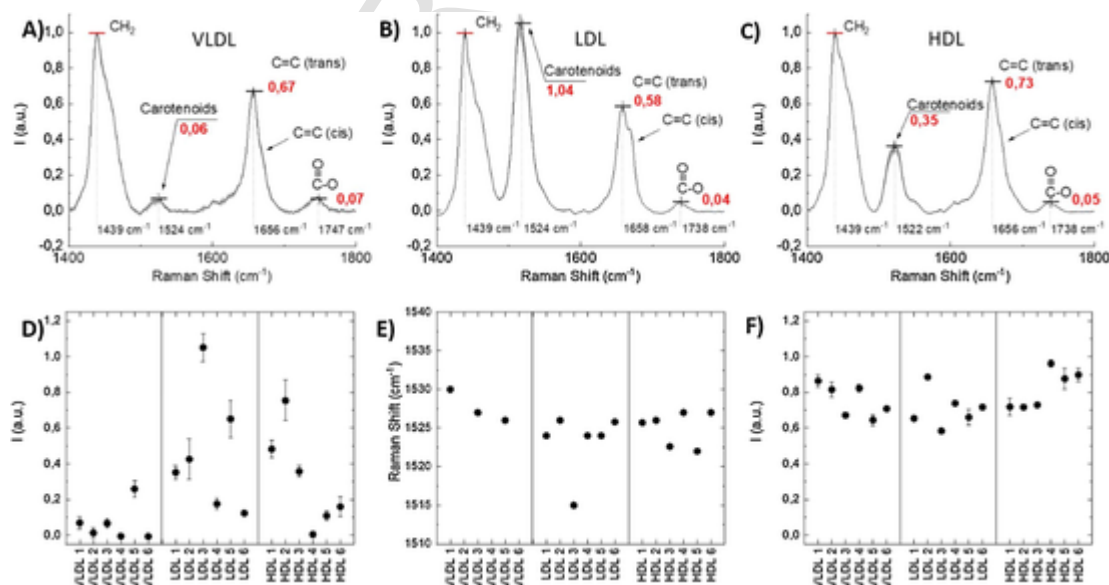


Fig. 6. Raman spectra acquired between 1400 and 1800 cm^{-1} on VLDL (A), LDL (B) and HDL (C) extracted from a single subject. The black line is the mean value of the five spectra acquired from each sample. The grey shadow represents the standard deviation of the five measure. D) Intensity of the bands of carotenoids at about 1524 cm^{-1} measured on VLDL, LDL and HDL of the six subjects included in the study. E) Exact peak position of the band of carotenoids on VLDL, LDL and HDL of the six subjects included in the study. F) Intensity of the peak at 1656 cm^{-1} measured on VLDL, LDL and HDL of the six subjects included in the study. In Fig. 4D-E black dots are the mean of the five spectra acquired on each sample, brackets are the standard deviations.

It is notable that even if cholesterol esters are also characterized by the presence of CH_2 chains, they do not have a peak at 1300 cm^{-1} (or it has a very reduced intensity). This was previously reported in the literature [25] and was clearly observed in the Raman spectrum of cholesteryl palmitate reported in the supplementary materials (Figure S1). For this reason, the peak at 1300 cm^{-1} can be used as a marker of the amount of triglycerides in LPs and was more pronounced in the triglyceride-rich VLDL (Fig. 5D).

Moreover, the shoulder observed at 1355 cm^{-1} is a band of the indole ring from tryptophan. The different intensity observed in VLDL compared to LDL and HDL might be due to a different exposure of the indole ring to the surface of the particles [29].

3.6. Region between 1400 and 1800 cm^{-1}

The last region of the spectra identified as particularly informative was the one between 1400 and 1800 cm^{-1} . Here, LPs had four main peaks at 1439 , 1524 , 1656 and 1738 cm^{-1} . The one at 1439 cm^{-1} of CH_2 scissoring vibrations appeared to be rather constant in all the acquired spectra and was selected as reference peak for the normalization of data. The band at around 1524 cm^{-1} of carotenoids, on the opposite, was highly variable between the classes of LPs and subjects. VLDL consistently had a very low, or absent, band of carotenoids, while LDL and HDL had bands with drastically different intensity (Fig. 6D). It is also notable to observe that also the position of the band of carotenoids changed between the subjects. This reflects the fact that the composition of anti-oxidants in LPs is not constant. For most of the analysed sample, the maximum of the carotenoids band was recorded at 1525 cm^{-1} , suggesting that beta-carotene is the most concentrated carotenoid in accordance with the scientific literature on the topic [30]. However, the shape of the band was not constant and, in some spectra, the maximum was shifted to 1530 cm^{-1} or 1515 cm^{-1} . The band at 1655 cm^{-1} of $\text{C}=\text{C}$ stretching vibrations allowed to detect the presence of unsaturated lipids. In fact, the ratio between the peak at 1655 and the one at 1439 cm^{-1} was reported as a method to measure the level of unsaturation in living cells [31]. This might be a particularly interesting finding as numerous studies focus on the different nutritional values of lipids with different level of unsaturation. A rapid and simple method to study how different nutritional lipids reflects on the unsaturation level of LPs could thus be of potential clinical interest. Even if our data are preliminary, we must notice that no clear difference emerged in the intensity of this peak at 1655 cm^{-1} between VLDL, LDL and HDL. Otherwise, it was highly variable between subjects.

The band at $1735 - 1750\text{ cm}^{-1}$ of $\text{CO}=\text{}$ stretching in esters was found in all classes of LPs and its intensity remained rather stable in all subjects. However, the position of this peak is shifted at 1750 cm^{-1} in LDL remarking the fact that they were mostly cholesterol esters, while HDL are constituted mostly by triglycerides and membrane lipids.

4. Conclusion

The aim of this study, performed on a limited number of healthy subjects, was to demonstrate how it is possible to obtain clear and reproducible Raman spectra from LPs extracted from plasma by ultracentrifugation in discontinuous KBr density gradient. The spectra obtained showed several peaks relative to the different biomolecules composing LPs and their intensity well reflects their relative composition. However, the Raman characterization of LPs is faster and requires significantly less work if compared with the traditional approaches used in lipidomics.

Since the composition of LPs is known to be altered in many pathological conditions, such as dyslipidaemias, this study paves the way for the application of RS in clinical nutritional studies and on a better understanding of several metabolic and pathologic conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vibspec.2020.103073>.

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