RAMAN STUDY OF THE SHOCKWAVE EFFECT ON COLLAGENS

José J. Cárcamo, Alvaro E. Aliaga, R. Ernesto Clavijo, Manuel R. Brañes and Marcelo M. Campos-Vallette*.

Laboratory of Vibrational Spectroscopy, Faculty of Sciences, University of Chile, POBox 653, Santiago, Chile.
*Correspondence to: Prof. Dr. M.M. Campos-Vallette. University of Chile, Faculty of Sciences, Chemistry Dept. POBox 653, Nuñoa, Santiago, Chile.
e-mail facien05@uchile.cl

Abstract

The Raman spectra (1800-200 cm^{-1}) of isolated dried collagens types I and III were recorded at different times after shockwave (SW) application in aqueous media. SW were applied in a single session. One week after the SW application the vibrational data analysis indicates changes in the conformation of the collagens; orientational changes are also inferred. During the next three weeks collagens tended to recover the conformation and orientation existing before SW application.

Keywords: Shockwaves, Raman, collagens, conformation, time dependence.

1. INTRODUCTION

Shockwaves (SW) are acoustic waves [1], transmitting pressure into materials through media such as water. Important improvements of shoulder rotator cuff tendinopathies are seen after shockwave (SW) treatment [2-3]; stimulation of neo-angiogenesis and hypercellularization are the result of short periods of SW treatment. SW are applied in a single session, releasing 4000 impulses of 0.3 mJ/mm^2 over affected areas in the shoulder. Recent developments in Raman spectroscopy are bringing to the forefront the possibility of using Raman scattering as a viable technique for proteomics and medical diagnostics [4-6]. We recently reported that immunohistochemical data indicate that collagen areas in tissues are influenced the most by SW treatment [7]. Thus, it should be relevant to study the effects of SW in aqueous media on the structure of isolated collagens.
Raman spectroscopy is an optimal tool for the structural characterization of collagen [8-9]. Raman spectroscopy is an optical technique that provides information about the molecular vibrations of any stable electronic state of the molecular system, and it is widely used for quantitative and qualitative studies in different research areas. Raman spectroscopy is a non-destructive tool for the structural/conformational characterization of biological systems with several advantages, such as high sensitivity to small structural changes, non-invasive sample capability, minimal sample preparation and high spatial resolution of the micro-Raman system [10]. This highly selective technique provides an impressive set of tools to tackle the problem of identification of the changes that may be induced by SW at the molecular level.

Collagens types I and III display structural differences mainly concerning the composition and length of the polypeptide α-chains. Collagen type I is composed of two α1 chains and one α2 chain; collagen type III is composed of three identical α1 chains. The collagen type III helix is slightly longer than in collagen I [11]. The polypeptide chains forming the collagen structural units in general contain mainly proline (Pro) (21%), glycine (Gly) (33%), alanine (Ala) (11%), 3- and 4-hydroxy Pro, and 5-hydroxylysine, linked together with a characteristic repeating X-Y-Gly pattern. Thus, one could expect that the Raman spectrum should be dominated by characteristic bands of these amino acids along with others coming from the protein conformation, that is, amide I, amide III and skeletal modes. Collagen structure and stability have been studied by Shoulders and Raines [12]. They demonstrated that stereoelectronic effects and preorganization play a key role in its stability, revealing in detail the fibrillar structure of type I collagen, the prototypical collagen fibril. Recently, Gullekson et al. [13] characterized through surface-enhanced Raman scattering (SERS) and tip-enhanced Raman scattering (TERS) the first layer of collagen molecules at the surface of the fibrils, by using Ag and Au nanoparticles bound to collagen type I fibrils, and tips coated with a thin layer of Ag. Some bands were assigned to phenylalanine and tyrosine. Amide band wavelengths suggested the presence of 3_{10}-helices as well as α- and β-sheets at the fibril's surface. Raman spectra of collagen fibers are known to show a marked dependence on the relative orientation of the fibers with respect to the laser polarization [8-9]. In particular, Bonifacio et al. [8] describe the effects of sample orientation in Raman microscopy of collagen fibers and their impact on the interpretation of the amide III band (1268-1245 cm^{-1} doublet).

The main objective of this contribution deals with the effect that SW in aqueous media have on the structure of collagens. We used Raman spectroscopy to draw inferences about the evolution time observed in the structures of type I and III collagens from rat and bovine after two hours, one week and three weeks following SW application. As a control we performed identical experiments without SW application. The present study should contribute to interpret the Raman spectra of collagen areas in human tissues before and after SW treatment.

2. EXPERIMENTAL

2.1 Shockwaves

The mechanisms for generating shockwaves (SW) are based on the Dornier method [1]. A high-energy electrical discharge across a spark gap is ignited in a water bath. Single SW were applied, releasing 1000 impulses of 0.15 mJ/mm^2 over collagen suspensions in plastic chambers containing cold sterile water. To this end we used a Duolith SD1 device (Storz, Germany).

2.2 Raman measurements

The Raman spectra were scanned with a Raman Renishaw Microscope System RM1000, with excitation at the 514, 633 and 785 nm laser lines, equipped with a Leica microscope and an electrically cooled CCD camera. Macro Raman measurements were obtained by using adequate macro accessories. The signal was calibrated by using the 520 cm^{-1} line of a

Si wafer and a 50x objective. The resolution was set to 4 cm^{-1} and 5 to 20 scans of 40 s each were averaged. Laser power was set in the 90 to 100% range, obtaining a maximum power on the sample of 1.5 mW. Spectra were recorded in the 1800-200 cm^{-1} region. Reproducible Raman spectra of collagens in the solid phase were scanned directly from the samples deposited on gold foils with the 785 nm laser line, thus attenuating or even quenching natural fluorescence. The spectral scanning conditions were chosen to avoid sample degradation. GRAMS/AI 8 software was used to analyse the spectral information.

2.3 Collagens

Solid collagen samples types I and III, from rat tail and bovine tail, purchased from Sigma and GenWay, were used as received without further treatment or purification. Samples, 3-5 mg, were suspended in 500 μl water and the whole was treated with SW. Raman spectra were scanned for the samples dried at room temperature two hours, one week and three weeks after SW application. In order to attenuate fluorescence, the samples were deposited on a gold foil. The same procedure was used for samples without SW application.

3 RESULTS AND DISCUSSION

Raman spectral modifications of type I and III collagens from rat and bovine after SW effect were evaluated from a structural view point.

3.1 Vibrational analysis. General information

The band assignment of the collagen samples is based on our Raman information and data from published spectral studies of simplest molecules such as tryptophan [14], lysine [15], cysteine [16], proline and valine [17], and complex molecular systems like collagen, gelatin and elastin [18]. Reported Raman spectra of collagens display medium/high peaks at 1669, 1452, 1269 cm^{-1} and a weak one at about 1000 cm^{-1}, the first corresponding to an amide I vibration [19]. Eleven bands were observed in the Raman spectrum of collagen by Lyng et al. [20], at 1655, 1447, 1299, 1243, 1082, 1060, 1032, 1002, 930, 854 and 813 cm^{-1}. Bands appearing at 1271 and 1248 cm^{-1} in the Raman spectra of collagen and elastin were assigned to the amide III mode; moreover, the Raman bands at 1668 and 1254 cm^{-1} and the weak one at 938 cm^{-1}suggest a mostly random structure for elastin [18]. Type I collagen has been identified in human skin through Raman micro spectroscopy [21]; amide I (1655 cm^{-1}), amide III (1246 cm^{-1}) and phenylalanine (1030 and 1004 cm^{-1}) bands of collagen were compared with data for the isolated molecule. We recently proposed a Raman band assignment for collagens which closely approaches the previously published data [7]. The proposed band assignment of collagens types I and III from rat and bovine is displayed in Table 1.

3.2 Raman spectra of collagens

The band assignments of collagens here proposed, Table 1, were made on the basis of the present results, data discussed in the previous section and related compounds [22-23]. Collagens types I and III display the amide I bands at about 1678 cm^{-1}, the amide III modes in the range 1280-1240 cm^{-1} and the skeletal vibrations around 940 cm^{-1}. These data

suggest a randomly disordered conformation for the collagens in the solid. Some amino acid bands are observed in the spectrum of collagens. This is the case for the bands of rat collagen type I at 1102, 942 and 859 cm^{-1} assigned to the δNCH, νCC skeletal and νCC ring modes of proline, respectively. The band at 1007 cm^{-1} is attributed to phenylalanine, while the weak band at 532 cm^{-1} corresponds to a $\delta CCN,COO^-$ mode of alanine. The weak bands in the collagen series at 879 cm^{-1} could be assigned to glycine.
Raman spectra profiles of collagens I and III from rat and bovine proved to be rather similar except for the 1350-1200 cm^{-1} region clearly observed in rat type III collagen, see Fig. 1.
Raman spectra of bovine collagens I and III are significantly similar, showing bands with identical frequencies and relative intensity profiles, see Fig.1. This is not the case for rat collagens I and III, where spectral differences are seen: the relative intensities of band pairs at 879-859 cm^{-1}, 942-925 cm^{-1} and 1250 cm^{-1} (type I bands are more intense than those of the type III collagen) and different frequencies for the bands of type I at 1678, 1459 and 1318 cm^{-1} with respect to their equivalents in type III. The spectra of bovine collagens type I or III are very similar to the spectrum of rat type I. These results indicate that the structures of bovine collagens types I and III are identical and that there is a conformational difference in the structures of rat collagens type I and III. Also, the structures of bovine collagens type I and III are similar to that of collagen type I from rat. Thus, the observed spectral differences could be ascribed to conformational aspects.
The amide I, amide III and skeletal bands are observed in all collagens at about 1670, 1250 and 940 cm^{-1}, respectively. This is interpreted in terms that all collagens display a predominantly random conformation [18].

3.3 Raman spectra of collagens without SW effect

Spectra of the four collagens were scanned at two hours, one week and three weeks without SW application. The resulting spectra for each molecular species remained unchanged. This spectral behavior is depicted for rat collagen type I in Fig. 2. The spectral behavior of the other collagens is available on request.

3.4 Raman spectra of collagens after SW application. Time dependence

The Raman spectra of the collagens recorded two hours, one week and 3 weeks after SW application exhibit several differences.
The spectrum of rat collagen type I evolves to another conformation that is detected two hours after after the SW treatment. Spectral changes are more evident at the end of the first week fromafter the treatment, see Fig. 3. The relative intensity of the bands at 1250 and 1275 cm^{-1} (amide III) change; these bands also display a slight frequency shift. It is possible that this spectral modification could be due to orientational changes following Bonifacio et al. [8]. Bands at 1476 cm^{-1} ($\delta NH,\delta NH_3^+$) and 595 cm^{-1} ($r,\delta,\omega COO^-$) appear or drastically increase their intensity. The intensity of the amide I band at 1678 cm^{-1} decreases and then remains constant until the end of the third week, shifting to slightly lower frequency. The collagen structure at the end of the third week tends to return to its original conformation that is nearly identical to that without the SW effect.
To verify the observed trends in the frequency and relative intensity variations we have fitted selected groups of bands, for instance in the spectral region 1400-1200 cm^{-1}, for all

the collagen systems. The intensity ratios of couples of bands and the spectral shifts are consistent with the qualitative issues. Band width variations are difficult to interpret on the basis of the present procedure.

The Raman spectrum of rat collagen type III changes two hours after the SW treatment: the relative intensity of the skeletal band at about 400 cm^{-1} and the νCN Pro band at 1036 cm^{-1} increase, while the bands at 1307 (δCH) and 1674 cm^{-1} (amide I) decrease in intensity. See Fig. 4. This situation is reverted one week after the treatment showing an equivalent spectrum to that without the SW effect by the end of the third week. The broad band at 1674 cm^{-1} remains with the same lower intensity.

Conformational changes are inferred for bovine collagen I at the first week after the SW treatment. Specifically, the relative intensities of bands at 922 cm^{-1} (νC-COO^-), 1101 cm^{-1} (δNCH Pro) and 1455 cm^{-1} (νCOO^-) change. The amide I band at 1674 cm^{-1} decreases in intensity; this modification could be associated to a molecular reorientation. These spectral changes, excepting the intensity of the band at 1674 cm^{-1} which remains weak, revert to the original appearance after three weeks.

In the case of bovine collagen type III, spectral changes were seen two hours after the SW treatment; the intensity of the δNCH band of Pro at 1098 cm^{-1} increases, the frequency shifts to lower energy (1091 cm^{-1}) and the intensity of the amide I band at 1672 cm^{-1} decreases, see Fig. 5. This spectral behavior is associated with conformational changes; a probable reorientation, leading to spectral modification of the amide I band intensity, cannot be discarded. At the end of one week and until the third week after the SW application the collagen reaturned to its original conformation. The final spectrum is identical to that without the SW effect.

At the end of the third week after the SW treatment the spectra of bovine collagens types I and III from bovine are almost identical. Rat collagens types I and III kept their original conformational difference. At the end of the third week after the SW application collagen conformations became identical to those without the SW treatment.

4 CONCLUSIONS

Raman spectral changes were observed in the relative intensities and widths of some specific bands of rat and bovine collagens types I and III after shockwave treatment. Spectra were recorded at 2 hours, one week and three weeks after the SW application. Our study indicates that the spectral changes appear within the first week after the treatment; then, the spectra evolve to the original spectrum as recorded without the SW effect. The spectral analysis allows us to conclude that the observed changes in the collagens are mainly originated by conformational modifications, without exclusion of possible orientational modifications, and that the new conformations appearing in the first week do not persist until the end of the third week. Conformation and orientation do not exclude each other. The collagen conformation and orientation evolved by three weeks returning to the original structural situation. These results are supported by identical experiments for all four collagens without applying SW, where no time dependence is observed in the spectra. This could be relevant to the interpretation of processes involved in the important improvements of rotator cuff supraspinatus tendon diseases observed after shockwave treatments, where neo-angiogenesis stimulation and hypercellularization also result from

short applications of SW. The present work is a first attempt to provide a structural approach of these bio-processes, most likely associated with biochemical changes originated by the shockwaves.

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FIGURE CAPTIONS

1. Raman spectra of (a) rat collagen type I, (b) bovine collagen type I, (c) rat collagen type III, and (d) bovine collagen type III.
2. Raman spectra of dried rat collagen type I without shockwave application in water after (a) 2 hours; (b) 1 week; (c) 3 weeks; (d) Solid.
3. Raman spectra of dried rat collagen type I after shockwave application. Time dependence in water, after (a) 2 hours; (b) 1 week; (c) 3 weeks; (d) Solid.
4. Raman spectra of dried rat collagen type III after shockwave application. Time dependence in water, after (a) 2 hours; (b) 1 week; (c) 3 weeks; (d) Solid.
5. Raman spectra of dried bovine collagen type III (a) 2 hours after SW, (b) 2 hours without SW effect.

Figure 1

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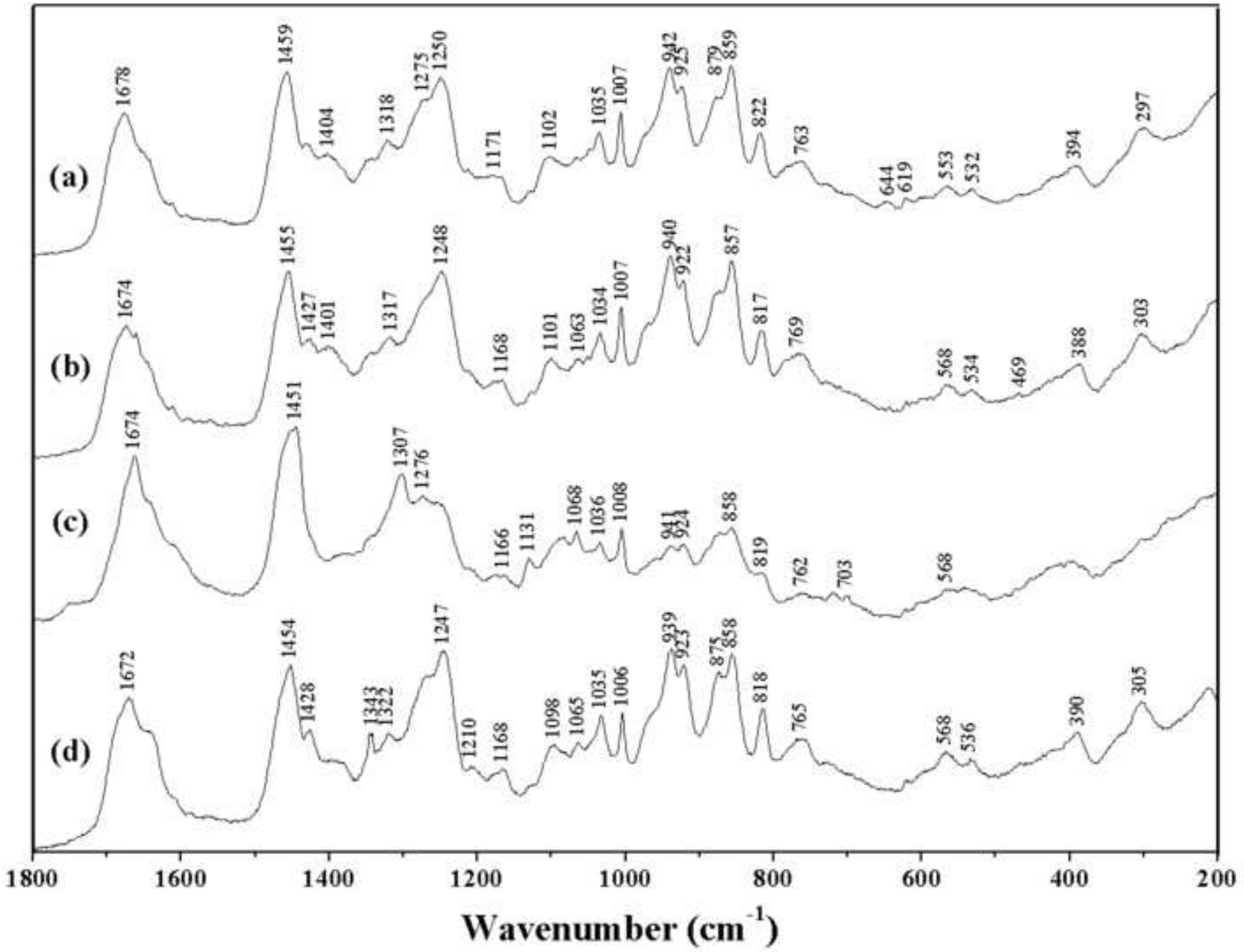

Figure 2

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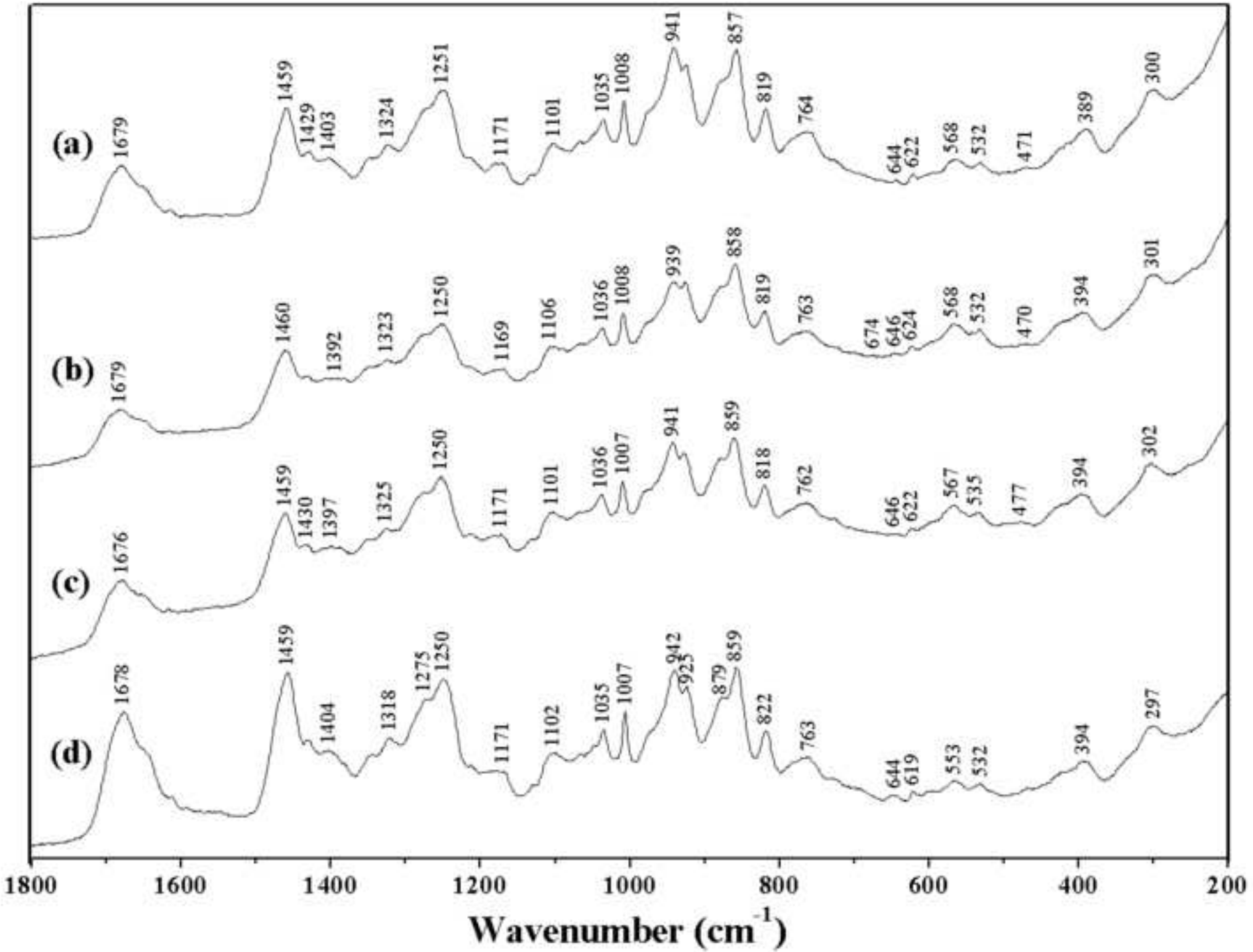

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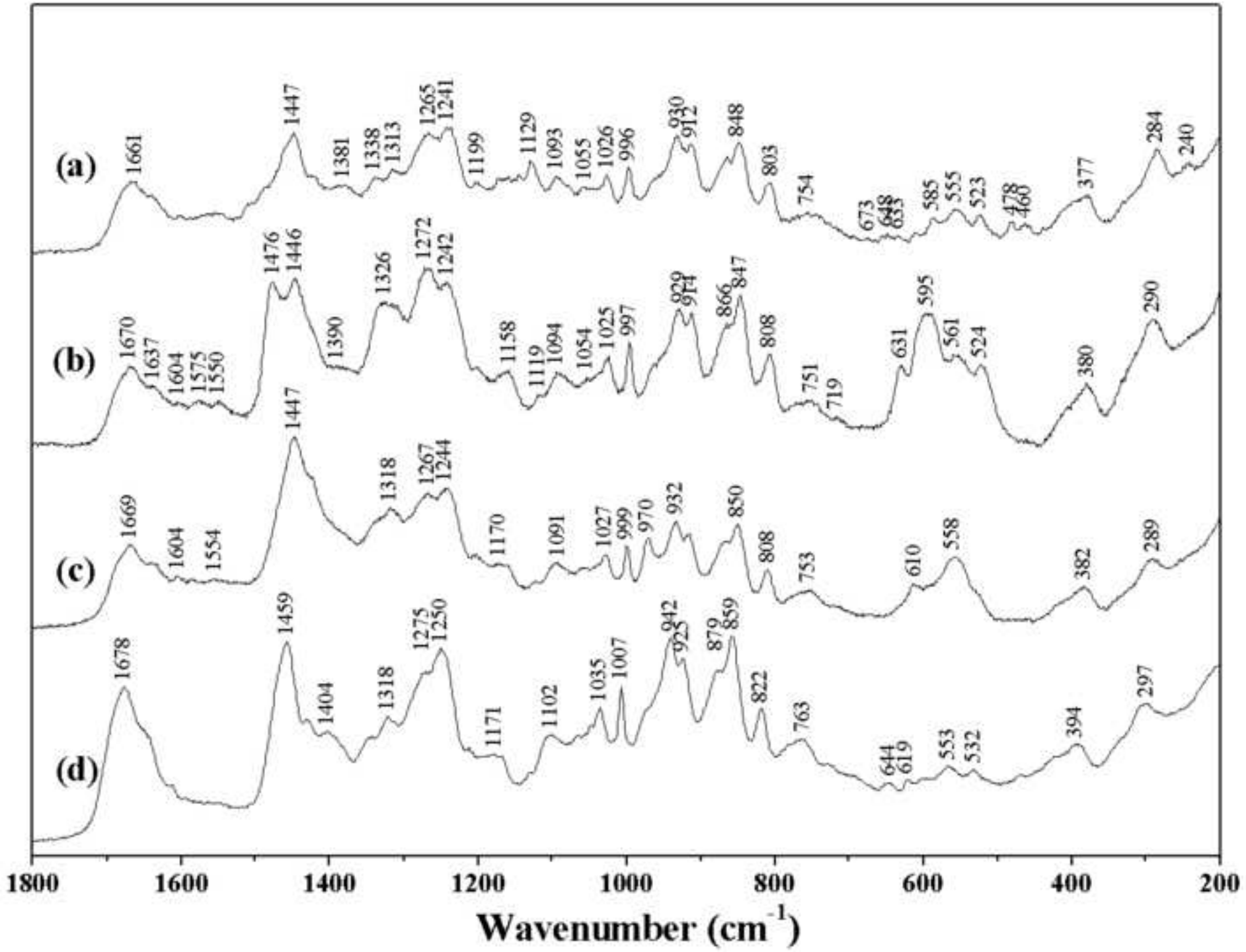

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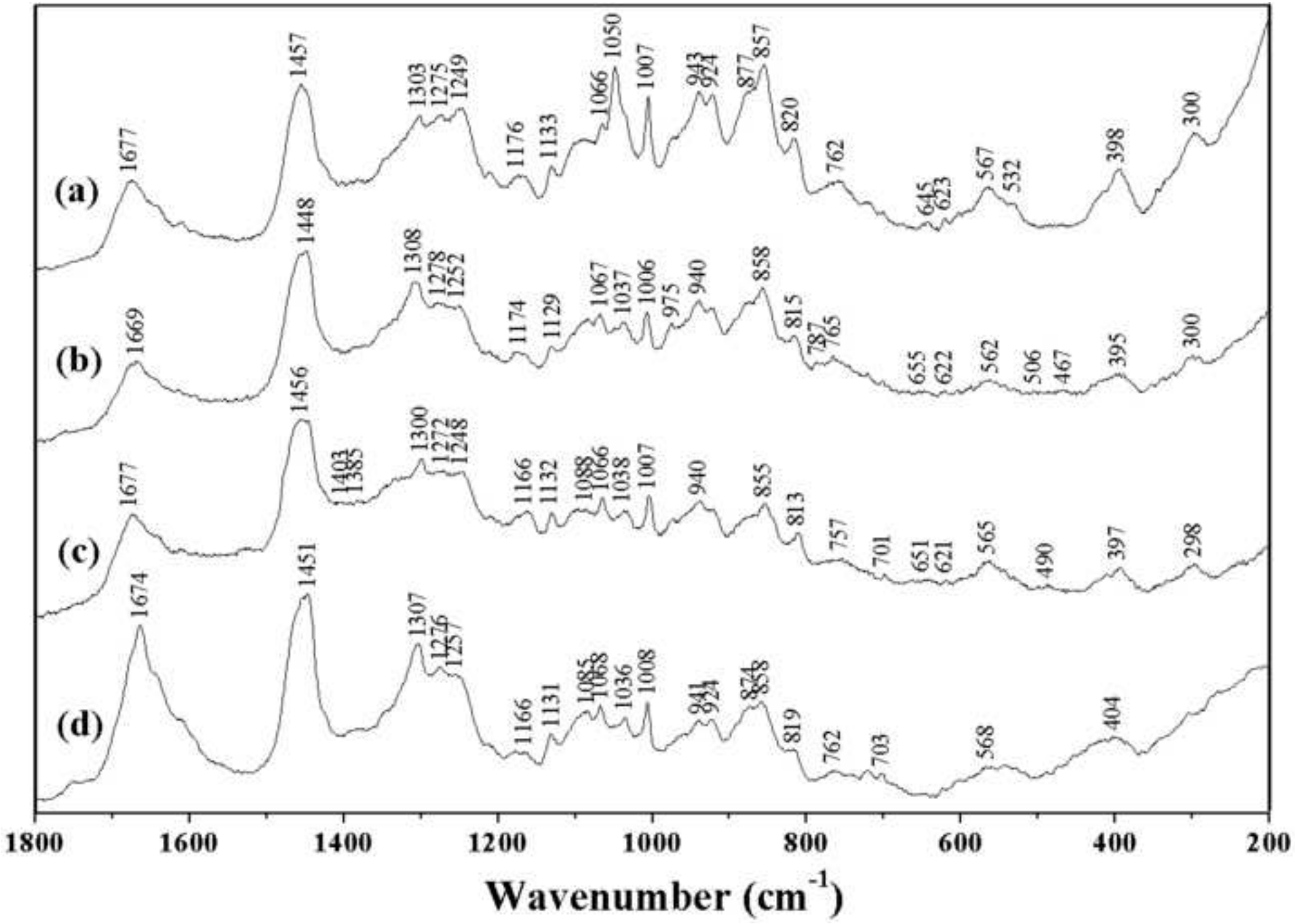

Figure 5

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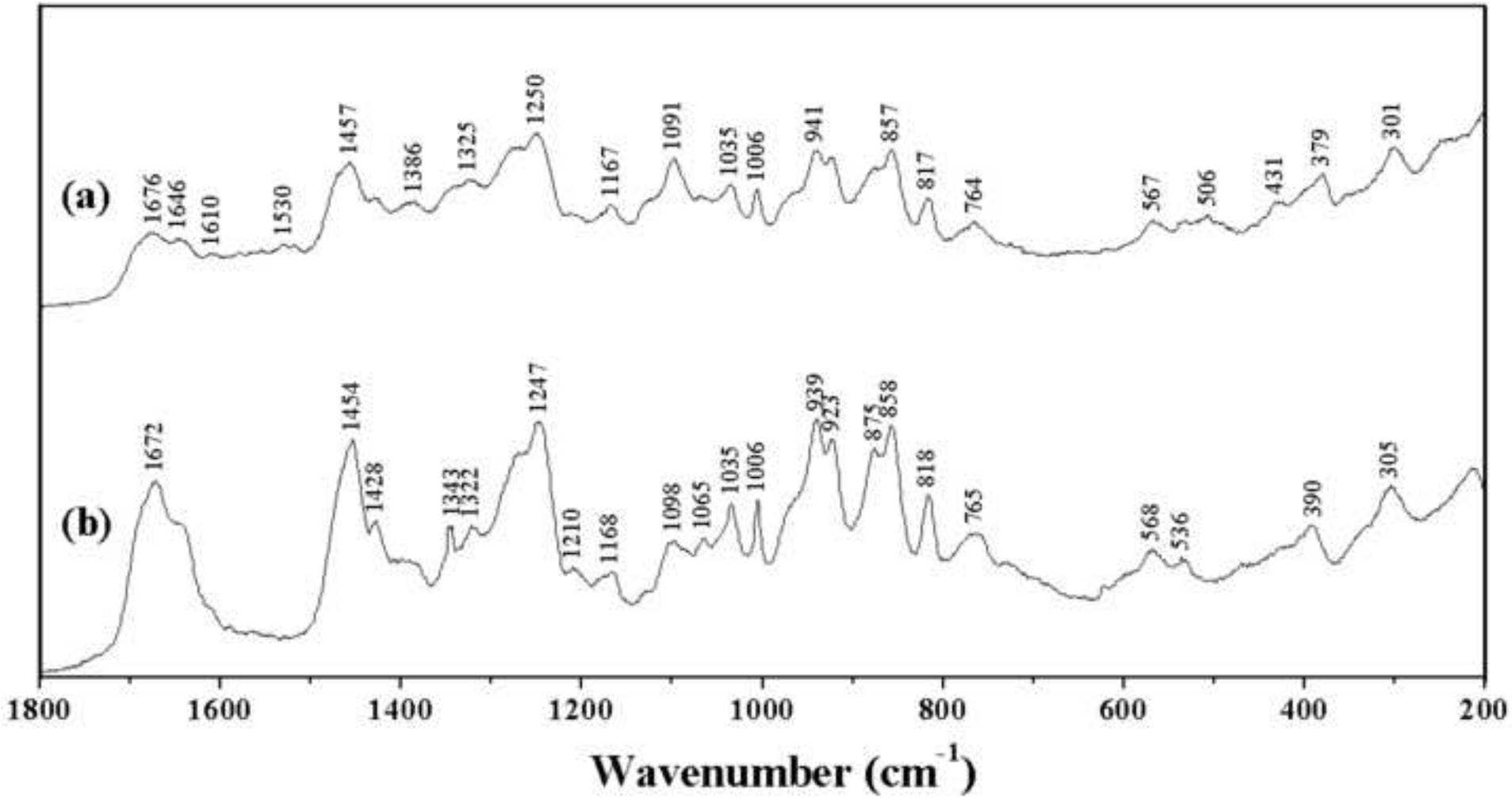

Table 1. Raman bands (cm^{-1}) in the range 1800-300 cm^{-1} of collagen type I and III from rat and bovine without SW application (A), with SW effect (B) and the most probable bands assignment.

A Collagen I rat	B Collagen I rat*	A Collagen III rat	B Collagen III rat**	A Collagen I bovine	B Collagen I bovine*	A Collagen III bovine	B Collagen III bovine**	Assignment
1678 m	1670 m	1674 s	1677 s	1674 s	1678 s	1672 s	1676 m	Amide I
							1646 sh	δNH_3^+, δNH_3^+
	1476 s							δNH,δNH_3^+
1459 s	1446 s	1451 s	1457 s	1455 s	1458 s	1454 s	1457 s	$\nu_{as}COO^-$, Lys
1429 vw				1427 w		1428 wsh	1429 w	δCH_3
1404 vw				1401 w	1401 bw	1401 bw	1386 mwb	ν_sCOO^-, Lys
						1343 m	1374 sh	ωCH_2 Gly
1318 w	1326 mb	1307 s	1303 w	1317 w	1349 vw	1322 w	1325 w	δCH
1275 sh	1272 s	1276 md	1275 w		1277 m	1269 w	1276 sh	Amide III
1250 vs	1242 sh	1257 m	1249 w	1248 s	1253 s	1247 s	1250 s	Amide III
1171 wm	1158 mw	1166 w	1176 mb	1168 w	1180 bw	1168 mb	1167 m	ωCH_2, tNH_2, rNH_3^+, hydroxylysine.
1102 bm	1094 mb	1131 w	1133 m	1101 mw	1101 m	1098 m	1091 s	δNCH Pro
1065 vw		1068 w	1066 w	1063 vw	1065 w	1065 w		νCN Pro
1035 vw	1025 m	1036 w	1050 s	1034 m	1038 ms	1035 m	1035 ms	νCN Pro
1007 m	997 s	1008 ms	1007 s	1007 ms	1007 s	1006 ms	1006 ms	Phenylalanine
942 s	929 s	941 vw	943 m	940 s	941 m	939 s	941 s	νCC skelet Pro
925 sh	914 sh	924 vw	924 m	922 sh	925 ms	923 sh	921 sh	νC-COO^-
879 sh	866 sh	874 sh	877 sh	876 sh	878 sh	875 sh	876 sh	νCC Gly
859 m	847 s	858 sd	857 s	857 s	859 s	858 s	857 s	νCC ring Pro
822 m	808 ms	819 w	820 m	817 m	819 ms	818 ms	817 ms	νCC skelet
763 wb	751 wb	762 vwb	762 bw	769 wmb	765 mb	765 mb	764 mb	δCOO^-
		703 w						δ,ω COO^-
644 w	631 wm		645 w		648 vw			r,δ,ωCOO^-
619 vw			623 w		622 w			
	595 sb							r,δ,ωCOO^-
553 vw	561 w	568 vw	567 m	568 w	567 mb	568 mb	567 mb	
532 m	524 w		532 w	534 w	535 m	536 w		δCCN, COO^- Ala
					467 vw		506 w	skeletal deform.
416 sh							431 w	
394 vw	380 mb	400 wb	398 msb	388 mb	391 mb	390 mb	379 ms	skeletal deform.
297 vw	290 mb	296 vw	300 msb	303 mb	302 mb	305 mb	301 ms	skeletal deform.

* After one week SW treatment

** After 2 hours SW treatment

Band description: w, weak; vw, very weak; wb, weak broad; mw, medium weak; md, medium double; ms, medium strong; sh, shoulder; s, strong.

Vibrational modes description ν stretching, δ deformation, ω wagging, r rocking.