BIOPHYSICAL TECHNIQUES REVEALED INSIGHT OF POTENTIZED SOLVENT OF ETHANOL-WATER INTERFACE

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Abstract. Over a five to six decades, biophysical techniques have played a very important role in the quantitative analysis of chemical, physical, and biological entities. This paper mainly focuses on quantitative analysis of structural aspects at atomic and molecular levels. In homeopathy, certain preparation of therapeutic components involves a serial dilution in the water-ethanol mixture used as potentization. The pharmacological action of medium-low potencies that contain low dose of active components entails high sensitivity in living system. There is enough evidence of their efficacy in several clinical studies, but their nature and mode of action are yet to be explained by scientific approaches. Several methods have been employed to understand the nature of the potentized homeopathic preparations, but the characteristics of these preparations are yet unclear. In the present investigation, we studied the characteristics of potentized preparations of sulphur using various biophysical techniques: UV-visible, fluorescent, and Raman spectroscopy. A bathochromic effect was observed in absorption spectrum of UV-visible spectroscopy of the samples of potencies from 4c to 8c ('c' denotes centesimal scale – dilution ratio 1:100), appearance of fluorescence properties and also a decrease in fluorescence intensity. Raman spectroscopy indicated a significant change in electronic configuration as well as the intermolecular interaction in liquid phase in potentized preparations. Therefore, on the basis of the obtained results, we assumed that some structural changes in potentized samples could take place.

Key words: Potentization, dilution, sulphur, Raman spectroscopy, UV spectroscopy, fluorescence spectroscopy, homeopathic medicines.

INTRODUCTION

In the homeopathic system, ultra-dilute potentized medicines are therapeutically used since the inception of homeopathy in the year 1796. The unique process of homeopathic medicine-preparation called 'potentization' entails the process of a) serial dilution and b) forceful striking (called succussion) and/or trituration.

The dilution level that will ultimately be used is beyond the Avogadro number [6]. It is often argued that the effects of homeopathic potentization are

Received: February 2020;
in final form March 2021.

ROMANIAN J. BIOPHYS., Vol. 31, No. 3, P. 107–120, BUCHAREST, 2021

either unspecific or obscure since common scientific theories and models cannot account for any measurable physical characteristics of the potentized dilutions. The preparations at concentrations below 6X (10^{-6}) ('X' denotes decimal scale – dilution ratio 1:10) have demonstrated the presence of source material and can be used for identification and standardization purposes [34].

In theory, repeated dilution steps leave progressively fewer and fewer particles of bulk from the original source of materials in true solution, until eventually none should persist in the solution after certain potencies. The standard chemical assays can identify lower number of particles from bulk source molecule for lower potencies. Higher potencies being more diluted cannot find any source molecules. As a result, medical fraternity deny the plausibility of pharmacological response to homeopathy dose, particularly at higher potencies. This also resulted in a lack of adequate evidence of the mode of action of the medicines thus prepared, which has kept homeopathy at distance from scientific acceptance.

In the recent years, the potentized preparations in 30c (10^{-60}) and 200c (10^{-400}) have shown to contain nanoparticles of the original starting materials (drug substances) of metallic [8] and plant origins [34]. Also, several studies have demonstrated the efficacy of the potentized preparations in *in vitro* studies [7, 9, 12, 17, 26], animal trials [21, 25] as well as in human trials [22, 23, 27] besides the clinical experience of the practitioners over two centuries. Previously, several working hypotheses [4, 15, 16, 35, 37, 38] have been developed revealing the mode of action of the potentized preparations, but none of them has been adequately validated so far, calling for a need for a deeper insight of the potentized preparations if the alteration in the physicochemical nature is due to dilution or succussion or both.

As the knowledge of the nature of the potentized preparations is yet insufficient, there clearly is a need for further research. One important step in the investigation of the physical properties of the potentized preparations using standard techniques. The vibrational and fluorescence spectroscopy are valuable tools for understanding molecular architecture in solution. In case of vibration spectroscopy, the transition between vibration states of the molecule is observed experimentally via infrared and Raman spectroscopy. These techniques provide important information about nature and chemical bond, intermolecular forces acting between atoms in molecules, and intermolecular forces in condensed phases. Similarly, fluorescence spectroscopy tool provides physical and chemical behaviour of macromolecules. The main advantage of this technique is its high sensitivity. Simply speaking, fluorescence consists in photon released from an excited state of a molecule. The excited state can readily be induced by the irradiation of a molecule in the ground state by specific wavelength of light.

Previous studies of physical properties of the potentized preparations included measurements of electrical conductivity, electrical resistance, dielectric constant, thermodynamic properties [11], thermoluminescence [19], and other methods such as nuclear magnetic resonance (NMR), spectroscopy and relaxation

[1, 2, 36] Raman spectroscopy and ultraviolet (UV) spectroscopy [32, 42, 43]. In a previous study, the differences in UV absorption of the potentized preparations and controls were observed [39].

In this study, the potentized samples of sulphur using various spectroscopic tools such as Raman spectroscopy, fluorescence spectroscopy, and UV visible spectroscopy were investigated.

MATERIALS AND METHODS

MATERIALS

Sulphur (purity 99 %), *Saccharum lactis* (lactose, $C_{12}H_{22}O_{11}.H_2O$) vehicle used in trituration process and ethanol (vehicle, 91 % v/v) were purchased from Sigma Aldrich, SB Lab., and Medilife Implex Pvt Ltd., respectively. Sterile water is used as a vehicle for dilution whenever necessary. All the reagents are of analytical grade.

METHODS

Potentization

As per the Homeopathic Pharmacopoeia of India (HPI), the process of potentization entails serial dilution and forceful striking (called succussion) or/and trituration. One part of original drug substance is mixed with 99 parts of the vehicle (alcohol or *Saccharum lactis* or water or water-ethanol mixtures) and exposed to ten powerful strokes through mechanical device, making 1c (centesimal) potency (10^{-2}). Again, one part of 1c potency is mixed with 99 parts of the vehicle, exposed to ten powerful strokes, making 2c potency (10^{-4}). Likewise, 30c (10^{-60}), 200c (10^{-400}), 1000c (10^{-2000}) and more are prepared. The preparations made using 1:99 and 1:9 proportions are labelled as centesimal (c) and decimal (X) potencies, respectively.

Potentized sulphur was prepared by triturating one part of pure sulphur with 9 parts of *Saccharum lactis* to arrive 1X potency. 1X to 6X potencies was prepared by trituration method and further liquid potencies from 4c to 100c were prepared using dispensing ethanol. Liquid potencies were potentized by means of a mechanical apparatus called potentizer. Standardized electromechanical potentizer [24] was used, imparting a torque of 404 N·m per potency for potentization of the samples. The first liquid potency of 4c was prepared by dissolving 200 mg of 6X into 100 mL of 1:1 ethanol-water mixture. After that, the successive potencies of 5c to 30c were made in ethanol (91 % v/v). Control sulphur samples were prepared as that of serial dilution method but were un-potentized.

Other controls used in the study were un-potentized ethanol (91 % v/v), potentized ethanol and pure sulphur. Un-potentized ethanol, potentized ethanol and ethanol-water mixture (1:1) were also studied. All the samples were studied in duplicate in three batches. The solutions were kept in special airtight stoppered dark-glass bottles to avoid evaporation and contamination.

Absorption study

The potentized preparations of sulphur from 4c to 10c potency and the controls were estimated by Implen GmbH Nano-Photometer/Spectrophotometer. The UV-visible absorption spectrum was recorded in the wavelength range of 200–500 nm using one cm path length quartz cuvette.

Fluorescence study

The changes in the fluorescence intensity of the potentized preparations of sulphur (1X, 5c, 10c, 15c, 20c, 25c, and 30c potency) and the controls were recorded in the wavelength range of 270–450 nm with an excitation wavelength of 260 nm on Varian, Cary Eclipse fluorescence spectrophotometer, at room temperature. Excitation and emission slit widths were set at 5 nm and the PMT voltage was set at 650 V using one cm pathlength rectangular quartz cuvette.

Raman spectroscopy

The potentized preparations of sulphur from 4c to 10c potency, pure sulphur, pure ethanol, and the controls were analyzed using an EZ Raman-xB portable Raman spectrometer (Enwave Optronics, Irvine CA, USA). The 785 nm wavelength laser, stabilized with narrow line having 250 mW instrument, allows recording of Raman spectra in the spectral ranges of 100–3,370 cm^{-1}. The optical spectral resolution was ~ 9 cm^{-1}.

RESULTS AND DISCUSSION

ABSORPTION STUDY

Ultraviolet-visible spectroscopy is one of the more ubiquitous analytical and characterization techniques in science. There is a linear relationship between absorbance and absorber concentration, which makes UV-Vis spectroscopy especially attractive for making quantitative measurements. Ultraviolet and visible photons are energetic enough to promote electrons to higher energy states in molecules and materials. UV-Vis spectroscopy is useful to the exploration of the electronic properties of materials and materials precursors in basic research and in the development of applied materials. Molecules containing π-electrons or

non-bonding electrons (n-electrons) can absorb energy in the form of ultraviolet or visible light to excite these electrons to higher anti-bonding molecular orbitals. The more easily excited the electrons, the longer the wavelength of light it can absorb. There are four possible types of transitions (π–π*, n–π*, σ–σ*, and n–σ*), and their transition energies can be ordered as follows: σ–σ* > n–σ* > π–π* > n–π* [18, 28].

The absorption peak of ethanolic sulphur is 264 nm according to Heatley [14]. In the present study, the absorption peak is obtained at 320 nm which shows the hypochromicity i.e., decrease in absorption spectra with bathochromic effect or redshift (increase in wavelength) as the potency increases from 4c to 9c (Fig. 1). Redshift is caused when the excited state is more polar as compared to ground state. Therefore, the polar solvents stabilize the excited state more than the ground state. Overall, there is a decrease in the energy gap between the excited and the ground state resulting in a redshift. The redshift observed in the control samples could be due to an increase in polarity of the samples since the volume of ethanol increases with each dilution. The hypochromicity is due to n → σ* transition of ethanol as a solvent. The potency of controlled samples beyond 8c cannot be plotted due to low absorbance value. It is interesting to note that absorption spectra of potentized sulphur samples for all the potencies are very low which is in accordance with Wolf [39] and therefore could not be plotted. This may be because of some physicochemical changes imparted by the process of potentization in homeopathy preparations. This preliminary hypothesis may further be investigated.

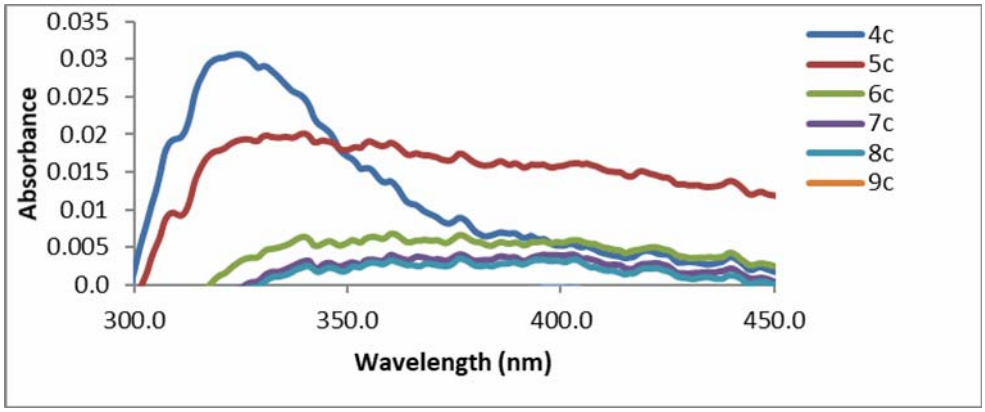

Fig. 1. UV-Visible spectra of control samples of sulphur from 4c to 9c potency.

FLUORESCENCE STUDY

Fluorescence spectroscopy, the most sensitive method with reproducible signals, can be quantified from the samples containing nanomole range of the

samples. The fluorescence signal can be analyzed in multiple ways including intensities, lifetime, energy, rotational freedom (polarization or anisotropy) to reveal various aspects of structure, interaction, mechanism or process. Moreover, the fluorimetry is a non-destructive method, so that any signal change can be determined as a function of time to determine its kinetics. Fluorescence signals are typically exhibited by polyaromatic compounds having conjugated π electron system. It occurs in gas, liquid, or solid chemical systems. The fluorescence is produced by absorption of photons in singlet ground states and promoted to singlet excited states. The spin of the electron remains still paired with the ground-state electron, unlike the phosphorescence. As the excited molecule return to the ground state, it involves the emission of photon of lower energy which corresponds to longer wavelength, than the absorbed photon molecular structure. Usually, the chemical environment affects the luminescence of a substance.

In the present investigation, the potentized and non-potentized samples of the ethanol-water mixture have exhibited fluorescence spectra (Fig. 2). When the ethanol-water (potentized and control) solutions were excited by ultraviolet light with the wavelength of 320 nm, the emission band was obtained in the range of (345–430) nm range. The emission peak is located around 345 nm. The decrease in fluorescence peaks was noticed in potentized samples of 10c, 20c and 30c. However, 1X potency did not show any fluorescence in both controlled and test samples. Pure ethanol (C_2H_5OH), pure water and pure sulphur (S_8 – octatomic cyclic structure), in un-potentized form, did not exhibit fluorescence spectrum.

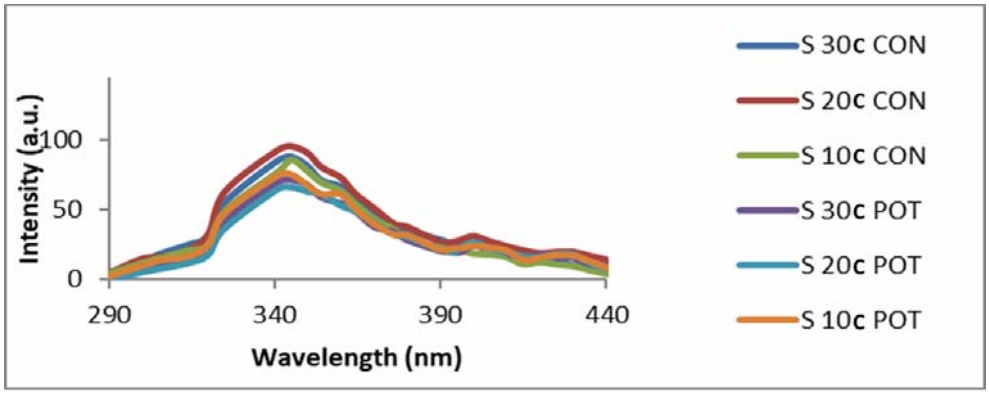

Fig. 2. Fluorescence spectra of potentized samples of 10c, 20c, and 30c potency and control; S = sulphur, CON = control, POT = potency.

It is reported that ethanol molecules conjugate with water molecules through hydrogen bonds to form molecular clusters, a ring or open chain structure, but conclusion is different from different experiments [30, 40]. The cluster structure of ethanol-water is still under investigation. In the study of Liu *et al*. group [5], the

combined number of ethanol-water cluster molecule groups were estimated using light-induced steady-state fluorescence spectra. They presented possible clusters whose fluorescence spectrum characteristics correspond to different frequency domain and time domain parameters. Thus, in the present result, it can be inferred that specific cluster can be created by the mixing of ethanol and water molecules which may cause emission of fluorescent spectrum. The molecular structure and chemical environment determine the fluorescent intensity of emission. Fluorescence occurs when an atom or molecules relax through vibrational relaxation towards its ground state after being electrically excited.

The decrease in fluorescence peak and emission in the sample of 10c to 30c could be due to some change in the potentized samples. In the process of potentization, a large amount of mechanical energy is transferred to the system (~404 N·m/10 strokes), causing a change in physical properties of the original material. Decrease in fluorescent intensity by a wide variety of process is a well-established phenomenon in fluorescence spectroscopy. The decrease in the fluorescent intensity is called quenching phenomenon. The quenching can occur by different mechanisms. Collision quenching occurs when the excited state fluorophore is deactivated upon contact with other molecules in solution, called quencher. Thus, further investigation is needed to understand the exact cause of a decrease in the fluorescent intensity in potentized samples, *i.e.*, mechanisms of the amount of mechanical energy transferred to the system during the process of potentization.

RAMAN SPECTROSCOPY

Raman spectroscopy is a versatile method for analysis of a wide range of samples through vibration mode of molecules [28]. This technique is commonly used in chemistry and biology to provide structural fingerprint by which molecule can be identified. Raman spectroscopy relies upon inelastic scattering of photons, known as Raman effect. Raman techniques referred for both qualitative as well as quantitative analysis of samples. Qualitative analysis can be performed by measuring the frequency of scattered radiations while quantitative analysis can be performed by measuring the intensity of scattered radiations [14].

Six peaks are simultaneously observed in the spectrum of pure ethanol (Fig. 5) in the region of 440–1,500 cm^{-1}. The peak at 1,468 cm^{-1} is due to the asymmetric deformation of CH_3 and the scissoring vibration of CH_2 whereas the peak at 1,280 cm^{-1} could be attributed to the twisting vibration of CH_2. In addition, the peak at 1,056 cm^{-1} is due to the stretching vibration of C–O. The most intense band is observed at 890 cm^{-1} and it could be attributed to the symmetric C–C stretch of ethanol.

Table 1

Changes in the intensity of Raman peaks of pure ethanol compared to the potentized and un-potentized samples (4c–10c)

Wavenumbers (cm^{-1}) of Raman peaks of pure ethanol	**Raman band assignment of pure ethanol**	**Changes in intensity of Raman peaks of un-potentized samples (4c–10c)**	**Changes in intensity of Raman peaks of potentized samples (4c–10c)**
450	Bending vibrations of C–C–O	Decrease in intensity	Increase in intensity with little broadening but less than pure ethanol
890	Stretching vibration of C–C	Decrease in intensity	Increase in intensity with little broadening but less than pure ethanol
1,056	Stretching vibration of C–O	Decrease in intensity	Increase in intensity with little broadening but less than pure ethanol
1,100–1,116	Rocking vibrations CH_3	Decrease in intensity	Increase in intensity with little broadening but less than pure ethanol
1,280	Torsion and rotational vibration CH_2	Decrease in intensity	Increase in intensity with little broadening but less than pure ethanol
1,468	Bending vibrations of CH_2	Decrease in intensity	Increase in intensity with little broadening but less than pure ethanol
2,884	Stretching symmetric vibration of CH_2	Decrease in intensity	Increase in intensity with little broadening but less than pure ethanol
2,932	Stretching symmetric vibration of CH_3	Decrease in intensity	Increase in intensity with little broadening but less than pure ethanol
2,977–2,985	Stretching asymmetric vibration of CH_3	Decrease in intensity	Increase in intensity with little broadening but less than pure ethanol

Peaks at 1,100–1,116 cm^{-1} are attributed to rocking vibration of CH_3. Finally, a broad and low-intensity band maximum at the 450 cm^{-1} is assigned to the deformation vibration of C–C–O [30]. There are other peaks at 2,881, 2,931 and 2,953 cm^{-1} assigned to stretching symmetric vibration of CH_2, CH_2 and CH_3, respectively (Table 1).

Table 2

Wavenumbers (cm^{-1}) of Raman peaks of pure sulphur compared to the potentized and un-potentized samples from potency 4c–10c

Wavenumbers (cm^{-1}) of Raman peaks of pure sulphur	**Raman band assignment of pure sulphur**	**Wavenumbers (cm^{-1}) of Raman peaks of un-potentized samples (ethanol-sulphur; 4c–10c)**	**Wavenumbers (cm^{-1}) of Raman peaks of potentized sample (ethanol-sulphur; 4c–10c)**
32	Not reported	32	32
165	E2 symmetric species of bending vibrations	150	150
230	A_1 symmetric species of bending vibrations	Disappeared	Disappeared
256	E_3 symmetric species of bending vibration.	278	278
448	E_3 symmetric species of bending vibration.	Disappeared	Disappeared
482	482 cm^{-1} is assigned to A_1 species of stretching vibrations	Disappeared	Disappeared

Pure sulphur (99 % powder) shows Raman bands at 165 and 230 cm^{-1} assigned to E_2 and A_1 symmetric species of bending vibrations. Peaks at 256 and 448 cm^{-1} are assigned E_3 symmetric species of bending vibration. A strong intense peak at 482 cm^{-1} is assigned to A_1 species of stretching vibrations [10, 31] (Table 2). Peak at 32 cm^{-1} has been observed in control as well as the potentized samples, however, this finding is new and not reported in the literature.

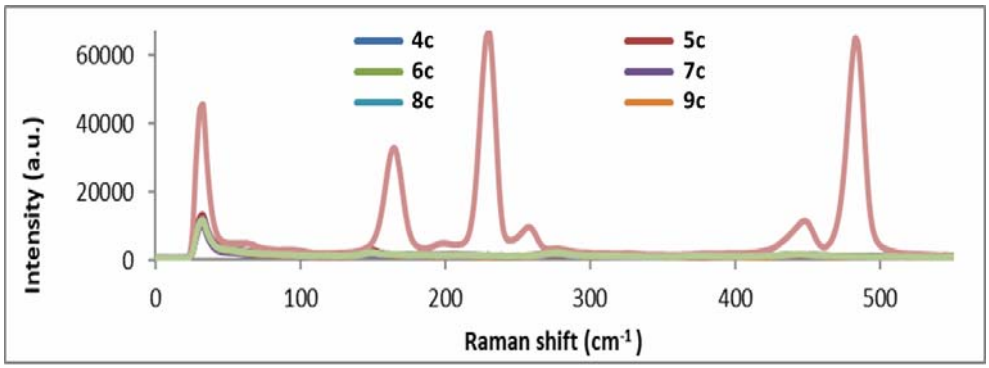

Fig. 3. Raman spectra of potentized samples from 4c–9c, pure sulphur, and pure ethanol.

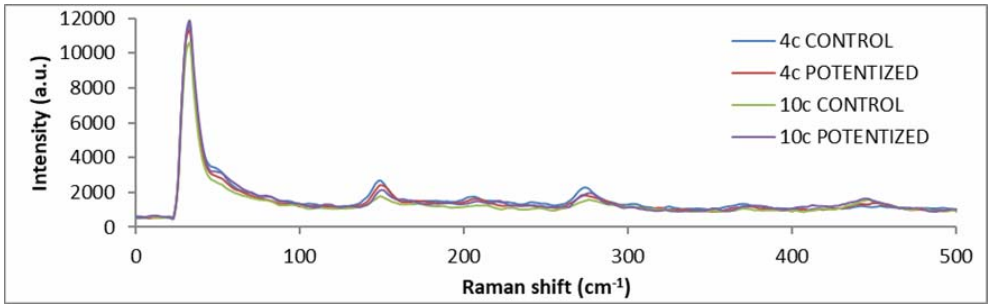

Fig. 4. Comparison of Raman spectra of control and potentized samples of 4c and 10c for sulphur region.

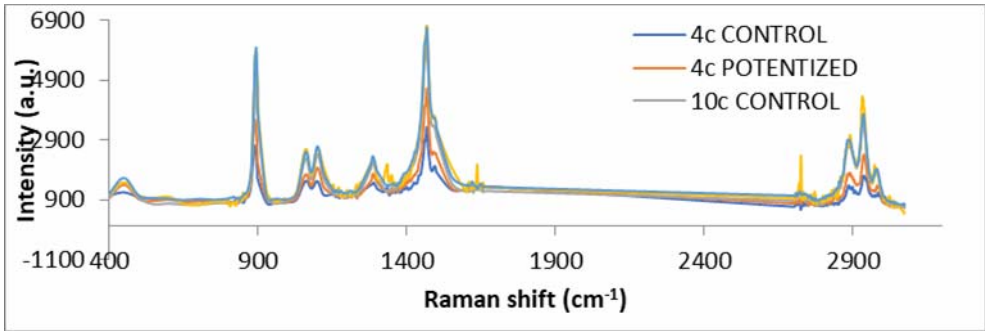

Fig. 5. Comparison of control and potentized samples of 4c, 10c and pure ethanol for ethanol region.

However, sulphur regions show the disappearance of a peak in the potentized sulphur samples compared to pure sulphur, which could possibly be due to the specific interaction of sulphur and ethanol which could not be comprehended (Fig. 3). There is variability in the controlled and the potentized preparations of sulphur in sulphur-band region indicating a change in the electronic configuration of sulphur (Fig. 4). There is an increase in the intensity and slight broadening of Raman peaks for ethanol band in the potentized samples compared to the controls (Fig. 5).

In general, the shape of line of spectra is determined by intrinsic properties of sample and further broadening may take place homogeneously or inhomogeneously. Homogeneous broadening is usually more extreme in solid samples due to morphology, local environment, chain length etc. The Gaussian confinement model takes place into account the contribution of the phonon away from the zone center to Raman phonon line shape proposed by Richter *et al.* [20] and has been extensively used by researchers. Arora *et al.* [3] reported in

theoretical models studied suggest that the confinement results in asymmetric broadening and a shift of the optical phonon Raman line, the magnitude of which depends on the width of the corresponding phonon dispersion curve. Therefore, in the present study we assume that the broadening and change in Raman intensity are due to a weak continuous absorption that follows the vibration band. This may be due to an interaction of the water molecule with ethanol groups, further broadening may be due to the confinement of optical phonon in the present sample. It is also possible due to the intermolecular interaction in liquid phase. Alcohol structure in liquid state is reported by Yu *et al.* [41]. Further broadening in Raman spectroscopic curves due to surface bond contraction and the quantum confinement were studied by Gao and Yin [13].

CONCLUSIONS

We investigated the potentized samples using biophysical techniques such as UV and visible spectroscopy, fluorimetry, and Raman spectroscopy methods. The UV transmission in the potentized preparations of samples were significantly lower than in the control. These observations clearly indicated changes in physico-chemical properties in potentized samples. The appearance of fluorescence in ethanol-water mixture is probably due to the formation of new molecular clusters. Similarly, a decrease in fluorescence intensity is an evidence of some changes in the molecules due to potentization of the samples.

Raman spectra showed a significant change in potentized sulphur preparations where a few peaks have disappeared with a few major shifts indicating a change in the electronic configuration of pure sulphur. There is an increase in the intensity and small peak-broadening in Raman spectra of potentized sulphur samples in comparison to the controls indicating a surface bond-contraction and a quantum confinement. Such microenvironment changes due to the process of potentization could be responsible for the biological action in this illustrative study using sulphur.

The experimental evidence indicates that the highly potentized homeopathic preparations, that is, diluted beyond the Avogadro limit, exhibit physicochemical properties different from the control of un-potentized samples. This could be possible because of an increase in the solvent's molecular dynamics for the potentized preparations. This also indicates that there is a significant interaction which is still unknown between sulphur and ethanol due to succussion. These differences in the physicochemical properties of potentized sulphur in ethanol clearly indicate that the presence of sulphur causes some modifications in solution. Both, the presence of sulphur as well as the process of potentization are responsible for the variation of the physicochemical properties of the potentized preparations. The lacking scientific method for distinguishing the physicochemical

nature of diluted preparation from the potentized preparation has been a major roadblock to biological research using the potentized medicines since the inception of homeopathy. This research finding could potentially stimulate more exploration in this direction.

*Conflict of interest***:** Authors declare no conflict of interest.

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