Published in "Journal of Raman Spectroscopy doi: 10.1002/jrs.3124, 2012"
which should be cited to refer to this work.

Quantification of purine basis in their mixtures at femto-molar concentration levels using FT-SERS

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Surface-enhanced Raman scattering spectroscopy represents one of the unique techniques for studying nanoscale objects, and its distinctive properties can be used in the process of further analysis. The careful evaluation of the particular influence of selected key-role experimental parameters (e.g. pH value of measured sample mixture, size and distribution of used nanoparticles) and the influence of reduction agent used in the process of formation of desired nanoparticle objects presents an important task in the further study of surface-enhanced Raman scattering effect. A broad study of these experimental parameters was performed in this paper. The main aim of the presented work was to a demonstrate an application potential of selected experimental conditions in the determination of three purine bases: adenine, xanthine, and hypoxanthine. The resulting limits of detection are at femtomolar concentration levels for all three studied compounds. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: FT-SERS; adenine; hypoxanthine; xanthine; Raman spectrometry; nucleotides; nanoparticle

Introduction

Nanoscale objects, e.g. nanoparticles, can be studied using distinctive approaches and by many different more common or even uncommon procedures. Common techniques used for the study of physical and optical properties usually comprise scanning electron microscopy,[1] transmission electron microscopy,[1–3] dynamic light scattering microscopy,[4] or by, especially in last few years, the very popular atomic force microscopy.[5] These techniques present a possibility to rapidly and easily characterize some of the key physical parameters of nanoparticles, e.g. their effective cross-section, density, surface characteristics or optical characteristics, respectively. Surface-enhanced Raman scattering (SERS) spectroscopy presents an interesting alternative substantially in the study of selected chemical properties of given nanoparticles at defined conditions.

Besides this assignment, SERS can also be employed in the study of various analytes of interest using nanoparticles as mediators for those actions. Signal enhancement using nanoparticles was described in detail, e.g. in the review by Smith *et al.*[6]

The study of experimental conditions is one of the key aspects of development of many methods utilizing SERS spectroscopy. The pH effect on surface charge and the resulting SERS spectra has been investigated using numerous chemical compounds, such as thiol molecules,[7–11] imidazole,[12] purine derivatives,[13] cytosine and uracil,[14] pyridines and acridines,[15,16] isonicotinic acid,[17–19] mercaptones,[20] humic substances,[21] lysozyme pigments,[22] dyes,[23] pesticides,[24,25] vitamins,[26] food additives,[27] nucleic acids,[28] amino acids,[29–32] proteins,[31,33–35] ligands,[36] lipids and lipolysis,[37–39] and drug components.[26,40–43] There even exist some studies on bacteria[44,45] that have investigated the pH effect.

The effect of nanoparticles cross-sections and size distributions has been also evaluated, e.g. in the work by Mahmoud and Badr,[46] where silver nanoparticles with various definite sizes were used. His investigation led to the important finding of nanoparticle size influence on the obtained enhancement factor and possible red shift. Another important work was performed by Glaspell *et al.*,[47] where nanoparticles in the range from 5 to 50 nm were studied, or by Nie *et al.*,[48] where single metal nanoparticles were evaluated. SERS effect was performed on Rhodamine 6 G. Link *et al.*[49] also investigated the influence of the size of nanoparticles used for SERS experiments; temperature dependency was also investigated in this work. It was shown that not only nanoparticle size plays an essential role but also the temperature of the measured solution has a significant effect on the resulting enhancement factor obtained by SERS measurements.

Detection or a respective determination of nucleotides, nucleosides or their free bases present one of the continuously increasing important analytical tasks especially in the field of biochemistry, medicine, and related branches.[50,51] Nucleic acids constituents present in the body fluids, tissues or cells can be products of nucleic acid catabolism, enzymatic degradation of tissues or unhealthy dietary habits.[52,53] Alternation of tracked concentration levels of these compounds can also indicate some substantial deviances of activities of selected catabolic, anabolic or interconversion enzymes. Nucleic acid constituents can thus in some cases present effective markers of various diseases that cause alternations in the purine and pyrimidine metabolic pathways.

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Analytical tasks dealing with an identification or determination of nucleic acids constituents are in most cases performed using capillary electrophoresis (e.g. Refs [54–60]), capillary electrochromatography[55] or high performance liquid chromatography (HPLC, e.g. Refs [61–65]). These techniques offer high sample throughput connected with automation and robustness, and are financially efficient. One of the main problems of these techniques lies in their comparably higher detection limits.

Sheng *et al*. published a method for the determination of selected purine basis using RP-HPLC using real-time SERS spectroscopy.[66] This method was based on the use of a separation technique (RP-HPLC) to separate target compounds and then, afterwards, SERS was used in an on-line detection technique. Analyzed concentration levels for target compounds were very good; they were in units of nmols per liter. This pilot work on an on-line approach introduced a possibility of using alternative analytical procedures (SERS) as detection systems. Development of methods targeted on the determination of selected analytes using SERS approaches present an interesting alternative to commonly used detection techniques but also have some significant limitations that have to be taken into account.

Du *et al*. developed a method based on SERS for the determination of melamine[67] and it can be seen that the limit of detection is in the order of hundreds of micrograms per liter. However, the experimental design presents a complicated step and also the analysis of real samples presents a limitation. Quantification of target analytes using SERS has also been discussed, e.g. in the works by Barber *et al*.,[68] Cai *et al*.,[69] Creighton,[70,71] Lindgren and Larsson,[72,73] and Xu *et al*.[72,73]

Koglin *et al*. described a method for the analysis of nucleic acid bases based on SERS effect established on the use of silver colloids.[74,75] This work brought interesting results but the method, in its original setup, was not able to quantify an amount of respective compounds.

The main aim of this work is to develop an easy to use and effective method for the determination of selected bases: adenine, xanthine and hypoxanthine (for details see the Fig. 1).

Experimental

Apparatus

All spectroscopic experiments were performed using a Fourier transformation infrared spectroscopy instrument Nicolet 6700 with NXR accessory (Thermo – Electron, USA). The instrument was equipped with a germanium detector cooled by a liquid nitrogen and Raman laser (NdYAG) with wavelength of 1064 nm. Laser power was set to 100 mW (on sample) and the aperture was set to 150 (fully opened). The setting of experimental parameters was performed using a standard solution of adenine ($c = 1*10^{-6}$ mol/L) and all the relevant experimental parameters (laser power, aperture, gain, optical velocity) were set to obtain the most intense and time independent SERS spectroscopy signal. All experiments were repeated five times if not stated otherwise.

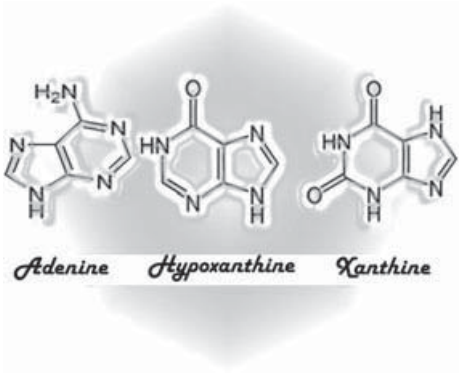

Figure 1. Schemes of analyzed compounds.

Chemicals

Adenine, xanthine, hypoxanthine, glucose, sodium citrate, and maltose (all pro analysis grade) were purchased from Sigma-Aldrich (San Jose, MA, USA). Silver nitrate, hydrochloric acid (36%), ammonium hydroxide (24%), and triethylamine (all pro analysis grade) were purchased from Fluka (part of Sigma-Aldrich, San Jose, USA). The water used was obtained from a Milli-Q water processing device (Millipore, USA) with conductivity less than 18 μS.

Preparation of standard solutions

Standard solutions of adenine, xanthine, and hypoxanthine were prepared by a precise and accurate weighting of 1 mg of the corresponding base standard. The standard was dissolved in 1 ml of deionized water giving concentration level of 1 mg/mL. This solution mixture was diluted to desired concentration levels defined by calibration range.

Preparation of nanoparticles

Nanoparticles were synthesized using a modified procedure described by Kvitek *et al*., [76] Soukupova *et al*.,[77] and Prucek *et al*.[78] This procedure is based on the reduction of silver salt (silver nitrate is usually used) by a selected reduction agent (e.g. reduction sugar, sodium citrate, etc.) in a basic environment. This work uses silver nitrate (concentration 1 mg/mL), where the silver is reduced using glucose ($c = 10$ mg/mL), maltose ($c = 10$ mg/mL) or citrate ($c = 10$ mg/mL). The basic environment resulted from the addition of ammonium hydroxide (0.1% v/v, pH = 10). Formed silver nanoparticles were stabilized using the addition of triethylamine ($c = 10$ mmol/L).

Preparation of working solutions

Working solution mixtures were prepared as follows. Precisely 200 μL of nanoparticles solution (60 ppm) was added to 690 μL of deionized water. Then, 100 μL of sodium chloride solution ($c = 100$ mmol) was added and the mixture was vortexed. Finally, 10 μL of analyte at corresponding concentration level was added. This solution was measured immediately.

Results and Discussion

The study of selected experimental parameters

First, SERS spectra for all respective compounds, namely xanthine, hypoxanthine, and adenine, were measured at the concentration level 1×10^{-13} mol·L^{-1}. The results can be seen in Fig. 2. It is clear that the resulting SERS spectra differ and regions that are unique for selected compounds can be easily found.

Second, selected experimental parameters were taken into account. It is clear that the reduction agent used has a strong

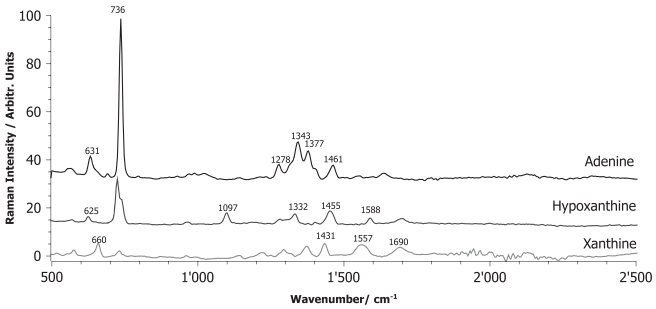

Figure 2. SERS spectra of adenine, hypoxanthine and xanthine measured at the concentration level 1×10^{-13} mol·L^{-1}.

influence on the resulting nanoparticle size and thus also on a significant number of the physical parameters by which the particle is defined. The main purpose of this work was to develop a SERS method for the analysis of selected nucleotides; thus, the influence of particle size on the resulting Raman peak areas has been evaluated. The obtained results are summarized in Table 1. It can be seen that the particle size (evaluated using dynamic light scattering microscopy) plays a significant role in the process of SERS spectroscopy measurements. Spectra obtained using glucose, maltose, and sodium citrate as reduction agents are shown in Fig. 3. It can also be seen that the use of sodium citrate and glucose led to similar results in contrast with the use of maltose that led to worse enhancement factors, outspread to this particular system and analytes. Another important parameter of the given nanoparticles is their stability in time. This factor was studied in the range of 10 days. Prepared nanoparticles were stored in the dark and at laboratory temperature for a given time and repeatedly used for SERS experiments (once every 2 days). The signal stability was studied. It was observed that the Raman signal decreases with time in the case of all selected silver nanoparticles. The study was based on the analysis of adenine ($c=1\times10^{-8}$ mol/L). After a week of use, the signal was at half of its initial value in all cases of used reduction agents. Obtained data are shown in Fig. 4. A significant decrease of Raman intensity in time can be seen for all prepared types of nanoparticles (maltose, glucose, and sodium citrate were used as reduction agents). According to this situation, nanoparticles were prepared every working day from this point.

Table 1. Summary of the obtained experimental values. The highest peaks in the corresponding spectra were evaluated. The average particle side (*d*) was obtained by dynamic light scattering microscopy

Reduction agent	Glucose, d = 44 nm		Citrate, d = 58 nm		Maltose d = 28 nm	
	AVG	**STD**	**AVG**	**STD**	**AVG**	**STD**
Hypoxanthine, peak area at 730 cm^{-1}						
PH 7	47.62	2.62	9.04	9.04	31.92	3.79
PH 10	40.85	2.48	14.41	3.86	18.48	5.12
Xanthine, peak at 660 cm^{-1}						
PH 7	65.44	1.48	20.54	3.46	17.11	2.67
PH 10	57.51	0.66	12.61	1.62	8.78	3.16
Adenine, peak at 736 cm^{-1}						
PH 7	199.49	9.75	115.45	12.39	180.59	3.16
PH 10	171.78	15.85	112.74	21.55	121.44	75.69

AVG, average of 5 experimental values; STD, standard deviation.

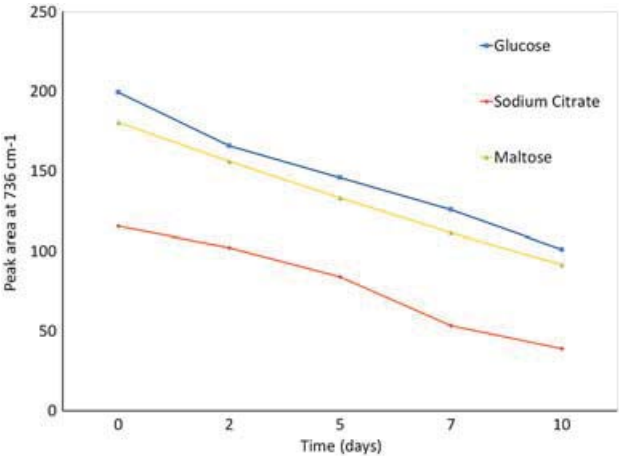

Figure 3. Dependency of selected Raman peak areas for respective compounds on time, $n=5$. Standard deviation of respective measurements is given by an error bar.

The third important parameter that was taken in account in this particular study is the influence of pH value of the measured sample mixtures. The influence of pH plays an important role in the case of proteolytic analytes, but has also some partial and not insignificant influence in other cases. It has been demonstrated that the state of a given analyte proteolytic balance influences the resulting SERS spectra mainly because a current proteolytic form of analyte, which is given by a pH value of environment, determines the method of its adsorption on silver nanoparticles.[79] The study of the influence of pH value was performed using three different pH values of sample mixtures, pH =3, 7, and 10. The value pH = 3 was obtained by the addition of hydrochloric acid ($c=50$ mmol/L), pH 7 was a native pH of the initial solution and pH 10 was obtained by the addition of ammonium hydroxide ($c=20$ mmol/L). For an illustration, Fig. 5 shows the analysis of adenine ($c=1\times10^{-8}$ mol/L) at different pH values. Similar results were obtained for xanthine and hypoxanthine (data not shown). It was demonstrated that the addition of hydrochloric acid leads to a suppression of SERS signal. According to our best knowledge, the basic principles of SERS effect are still unknown but it can be seen that a shift of proteolytic balance can significantly influence a desired Raman spectrum. One of the possible causes can be the presence of positive charge on the heterogeneous nitrogen atom by which an analyte is adsorbed on a silver nanocomposite. On the other hand, higher pH values led to a significant increase of Raman signal, which supports our theory.

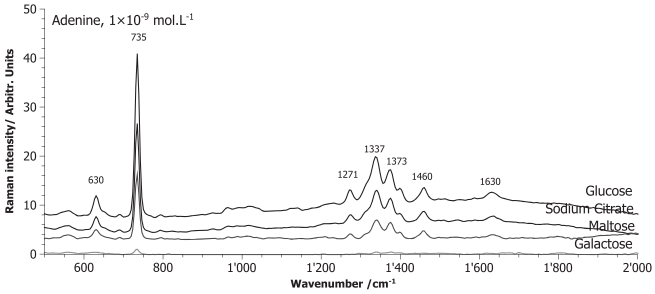

Figure 4. SERS spectra of adenine obtained for experiments where various reduction agents were used during the preparation of concrete nanoparticles.

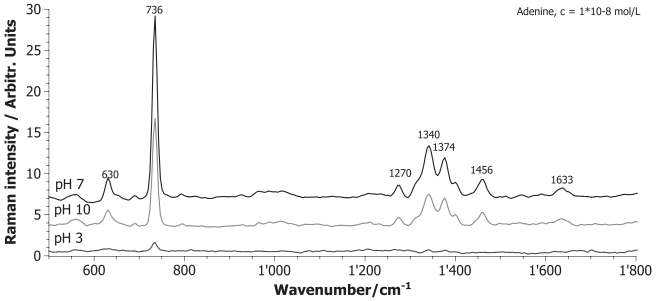

Figure 5. Influence of pH value of analyzed sample mixture on corresponding SERS spectra.

According to the results that were obtained during the study of experimental conditions, final conditions were selected and consequently used in the study of calibration parameters. The highest intensity of respective SERS signal was obtained with glucose as reduction agent of silver nitrate. Particles prepared using the mentioned reduction agent embodies suitable properties concerning the Raman enhancement factors and their stability in time. The best results were obtained using a pH value at pH = 7.

All obtained experimental values are summarized in the Table 1. The influence of selected experimental parameters, i.e. pH and reduction agent, was evaluated using peak area of the highest peak in the corresponding Raman spectrum. For adenine, the peak at 735 cm^{-1} was selected. This peak corresponds to a vibration of the C = N bond in the purine ring. For hHypoxanthine, the peak that corresponds to a similar vibration was selected. It was located at 730 cm^{-1}. In the case of xanthine, the peak at 660 cm^{-1} was selected. This peak was interpreted as the C = N vibration on the pyrimidine ring.

Calibration model design and evaluation

The quantitative analysis was based on the evaluation of SERS spectroscopic data. For each target analyte, the highest peak in the corresponding spectrum was selected and taken into account. Its known that this approach is valid only in the narrow concentration rang and thus only a narrow interval of concentration levels was used.[80] This limited validity is given mainly because of the concurrent adsorption of ballast species on the nanoparticle surface and by their limited surface (higher limit), and on the other side of the range there can be found serious problems with excitation laser incoherence and instrumental noise. However, it is possible to obtain qualification information using SERS experiments by a careful selection of experimental conditions and using defined and highly restricted concentration range. In an agreement with the mentioned possibilities, every evaluated spectrum was compared with the corresponding spectrum obtained by the analysis of a blank solution (working solution without an addition of analyte). In this way, the target peak in the analyte spectrum was compared with a corresponding peak in the blank spectrum and these two peaks were abstracted. The obtained reduced peak area was afterwards used for further evaluation. Every calibration level was measured five times if not stated otherwise.

Calibration levels for target compounds (adenine, xanthine, and hypoxanthine) were selected in the concentration range from 1×10^{-15} mol/L to 1×10^{-8} mol/L in ordered intervals (eight calibration levels). However, the linearity of calibration curves was obtained only in the lower part of this interval. For adenine, the linearity of the calibration curve was obtained in the range from 1×10^{-15} to 1×10^{-11} mol/L; for hypoxanthine and xanthine, this interval was from 1×10^{-14} to 1×10^{-10} mol/L, respectively.

The curvature of the calibration curves at higher concentration levels could be caused, e.g. by concurrent adsorption processes on the limited surface of nanoparticles described above.

The limits of detection (LOD) were calculated from the obtained spectra. Measured data were compared with corresponding blank spectra and after subtraction, LOD was calculated from the highest peak in the given reduced spectrum using an approach that is based on the signal-to-noise ratio. The summary of the obtained results can be seen in the Table 2.

Table 2. Summary of the obtained results of the calibration experiment. Calibration is based in a linear model ($y = Ax + B$)

Calibration; $y = Ax + B$						LOD
	A (mg/L)	STD	B (mg/L)	STD	R^2	mol/L
Xantine	0.82	0.01	3.44	0.21	0.99	2×10^{-15}
Hypoxantine	1.82	0.02	213.74	1.25	0.92	1×10^{-14}
Adenine	2.82	0.01	0.04	0.01	0.99	1×10^{-15}

STD, standard deviation.

Analysis of model mixtures

The analysis of model mixtures was based on the similar approaches that have been used in the calibration model design. Samples containing all three target compounds on the selected concentration levels were measured. These concentration levels were selected according to the results that were obtained in the process of calibration model development. For all three target compounds (adenine, xanthine, and hypoxanthine), these levels were 1×10^{-12} and 1×10^{-13} mol/L of the respective target compound. A new mixture calibration model has also been constructed. This calibration model was based on the analysis of the mixture containing all three targets: xanthine, hypoxanthine, and adenine. The calibration range was selected from 1×10^{-15} to 1×10^{-10} mol/L. For each target compound, its unique peak was found. The selection criteria for these peaks were based on two prerequisites: first, the selected peak has to be unique for a target compound and second, this peak has to be intense enough for quantification purposes at femtomolar concentration levels. For adenine, the peak at 735 cm^{-1} was used; for hypoxanthine, the peak at 1097 cm^{-1} was used; and for xanthine, the peak at 660 cm^{-1} was selected. On the basis of these principles, calibration curves for each target compound were constructed. Calibrations were linear in the whole selected ranges for all three compounds. The spectrum obtained by the analysis of the sample containing 1×10^{-12} mol/L of target compounds can be seen in the Fig. 6. Two model samples containing 1×10^{-12} and 1×10^{-11} mol/L of all target compounds were prepared to test the calibration. Results obtained by the analysis of these model samples are given in the Table 3. It can be seen that the analysis of sample mixtures brought some difficulties that were reflected in a relatively lower obtained correlation coefficients (compared with an analysis of standards), but the method is suitable for a screening analysis of selected compounds in the mixture on very low concentration levels.

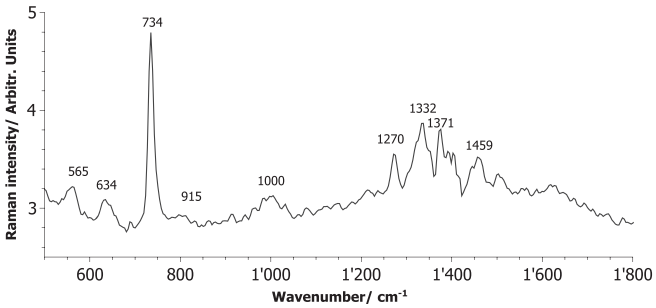

Figure 6. SERS spectrum of the mixture of target analytes: adenine, hypoxanthine, and xanthine, $c = 1 \times 10^{-12}$ mol/L.

Table 3. The summary of results obtained by an analysis of model sample mixtures, RSD stands for relative standard deviantion.

	Sample A [mol/L] 10^{-13}	RSD [%]	Difference [mol/L] 10^{-13}	Sample B [mol/L] 10^{-13}	RSD [%]	Difference [mol/L] 10^{-12}
Adenine	9.5	4.5	0.5	98	5.1	2
Xanthine	7.2	5.5	2.8	105	5.2	5
Hypoxanthine	14.9	4.8	4.9	108	4.8	8

Concluding Remarks

A method for the analysis of model sample mixtures of selected nucleotides was developed. This method is based on the use of SERS spectroscopy with Fourier transformation. Adenine, hypoxanthine, and xanthine were selected as model analytes. The obtained limits of detection are in the units of femtomols for adenine and xanthine and in tens of femtomols for hypoxanthine. By the analysis of model sample mixtures containing all three selected compounds, it was shown that the obtained RSD is on average 5% (from 4.5% to 5.5%) for both selected model samples.

Acknowledgements

The authors gratefully acknowledge the support of the Operational Program Research and Development for Innovations - European Regional Development Fund (Project No. CZ.1.05/2.1.00/03.0058 of the Ministry of Education, Youth and Sports of the Czech Republic).

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