

Method and Apparatus for analyzing a liquid sample by Raman Spectroscopy

Field of the Invention

- 5 The present invention relates to analyzing a liquid sample by Raman spectroscopy. It particularly relates to techniques in which the liquid sample comprises multiple analyte components in a liquid medium.

Background of the Invention

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Raman spectroscopy is a spectroscopic technique widely used as an analytical tool in the chemical sciences. It relies on the “Raman effect”, inelastic scattering of monochromatic light, usually from a laser in the visible, near infrared, or near ultraviolet range. The laser light interacts with molecular vibrations, phonons or other
15 excitations in the system, resulting in the energy of the laser photons being shifted up or down. The shift in energy gives information about the phonon modes in the system.

The Raman effect is small (it shifts the energy of only about 1 in every 10^9 photons in the incident laser light), so Raman spectroscopy is most easily performed with pure or
20 concentrated substances. Raman spectroscopy is frequently used to identify the primary chemical constituents present in a macroscopic sample, and/or for quality control. Examples would be measurements of an organic powder (an active pharmaceutical ingredient), an inorganic powder (the pigment TiO_2), minerals, organic solvents etc.

25 It was discovered about 30 years ago that the Raman effect may be increased by a factor of 10^{10} - 10^{15} if a molecule is on or near a silver or gold surface. This new phenomenon was called Surface Enhanced Raman Spectroscopy (SERS). SERS opens the possibility to measure trace analytes. Thus, for example, if there is an impurity in water, a drop of impure water can be deposited on a silver surface, the water evaporated, and then the
30 impurity measured – even though the impurity might be on the micro- or even nano-gram scale. SERS is normally performed with a small substrate (or “chip”) which has a

silver or gold metal surface, and the drop of liquid is placed on the surface of the SERS substrate.

5 A complex molecule with many functional groups will have a very large number of vibrational bands, so that the Raman spectrum of even a single analyte can be very complicated. If a liquid sample contains multiple analytes the spectra will typically be overlapping, giving the sample as a whole a complex Raman spectrum. In most experimental situations the analytes are not known in advance, and they are not available in a pure form. For this reason, it may be essentially impossible to determine
10 which analytes have contributed to the Raman spectrum of the liquid sample, so the SERS analysis fails.

Although no satisfactory technique exists for performing SERS analysis of a liquid sample with multiple unknown components, this is not true of all applications of Raman
15 spectroscopy. In the case of solid samples, it has been proposed to use a BTEM (Band Target Entropy Minimisation) algorithm to untangle individual pure component spectra from multi-component mixtures.

BTEM is a blind-source-separation algorithm, which does not need to have any a priori
20 information. Instead, BTEM needs a number of distinctly different spectra as input, each containing the pure component spectra in different proportions. The BTEM extracts the pure component spectra. It is thus a "self-modeling" curve resolution technique. The BTEM technique has been successfully used to analyze data from FTIR (Fourier transform infrared spectroscopy), UV-VIS (Ultraviolet-Visible spectroscopy),
25 NMR (nuclear magnetic resonance) spectroscopy and MS (mass-spectroscopy). When BTEM is applied to Raman spectroscopy it is typically in situations in which a solid sample contains certain chemical components in different proportions in different locations within the solid sample. For example, a sample of pottery typically contains slightly different proportions of impurities in different locations. At first sight, it would
30 not be expected that this prior art technique (Raman plus BTEM) would be successful for analyzing a homogeneous liquid sample, since there is no intrinsic variation in the signals present in a set of measurements (or from point to point).

Summary of the invention

5 The present invention aims to provide methods and apparatus for analyzing a liquid sample containing multiple unknown analyte components.

10 In general terms, the invention proposes that the liquid sample containing multiple analytes is used to produce a layer on a SERS surface (which may be a surface of a single SERS substrate, or the collective surfaces of multiple SERS substrates, or a surface of any other SERS device, such as SERS nanowires) and that multiple
respective Raman spectra are collected from each of multiple locations on the SERS surface. Each of the Raman spectra comprises multiple signal components which are pure component spectra from the corresponding multiple analytes. The different Raman spectra have different respective ratios of those signal components. The multiple Raman
15 spectra are used to obtain information about the analytes.

A first possible reason that the Raman spectra contain different respective ratios of signal components from the multiple analytes is that the analytes are present in different proportions in the different locations. That is, the layer may be inhomogeneous.
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Another possible reason is that, even if the analytes are present in the same proportions in the different locations, the SERS surface is different in the different locations, leading at each location to the analytes generating Raman scattering in a different ratio. That is, the analytes may interact with the SERS surface in different ways at the different
25 locations. For example, in the case of two analytes, a first of the analytes may generate more Raman scattering than the second analyte at a first location, and less Raman scattering than the second analyte at a second location, even though the concentrations of the first and second analytes are the same at both locations.

30 A self-modeling curve resolution technique such as BTEM may be applied to the multiple Raman spectra to extract the multiple pure component spectra. The pure component spectra may be used to identify the analytes, such as by comparison with a

reference library of one or more pre-known pure component spectra (which may have been originally obtained by experiment, or be a SERS spectrum, predicted from first principles, of a species believed to be present as an analyte). Alternatively, there are possible applications of the invention without using pre-known spectra. An example
5 might be in a situation in which it is desired to make sure that there are no chlorinated organics in a water supply. If an embodiment of the method shows that the water supply contains an analyte having a pure component spectrum with a strong band at circa 550-800 cm^{-1} (indicative of C-Cl vibrations) and other at circa 800-1000 cm^{-1} (indicative of C-C vibrations) and distortions at circa 1200-1500 cm^{-1} (indicative of various C-H
10 vibrational modes), then this suggests that an organic chloride is present, and there is no need for a reference library.

Two exemplary techniques for producing the layer are now explained, both exploiting physical effects which, so far as the present inventors are aware, were not previously
15 known, or at any rate known to be useful for a SERS process.

Firstly, the liquid sample can be evaporated onto the SERS surface, and then respective Raman spectra are obtained from areas which are of microscopic size. These areas can be non-overlapping or partially overlapping. The present inventors have discovered that
20 typically on a microscopic scale the deposited analytes are heterogeneously distributed and/or the ratio of signal contributions are heterogeneously distributed. Typically, the regions have a maximum diameter in the range about 1 micro to about 20 microns, though slightly smaller and larger sizes are possible. The maximum diameter might for example be no more than 5 microns, or no more than 2 microns. A Raman microscope,
25 with spatial resolution of about 1 micron, can provide hundreds or thousands of distinct spectra from one sample.

Secondly, as mentioned above, the SERS substrate(s) may be formed with a surface which is inhomogeneous. This inhomogeneous surface may improve the inhomogeneity
30 of the deposited analyte layer which is produced during the evaporation process and/or create inhomogeneous levels of Raman scattering. The inhomogeneity may be spatial (e.g. surface roughness or structure) and/or chemical (i.e. atomic composition).

The inventors have discovered that analytes in a liquid sample are adsorbed onto the inhomogeneous surface in different proportions on such a surface and/or the ratio of signals contributions are inhomogeneously distributed. The use of an inhomogeneous surface thus makes it possible to obtain the layer on the SERS surface containing the spatially different proportions of analytes, even without evaporating the liquid of the sample away. The Raman spectra can be collected by Raman spectroscopy of the adsorbed layer.

- One possibility is for a SERS substrate to be employed which has different areas having spatially-differing surface. A technique for fabricating such a SERS substrate with a inhomogeneous (but regular) three-dimensional surface is disclosed in R.Z. Tan et al "3D arrays of SERS substrate for ultrasensitive molecular detection", Sens. Actuators A: Phys. (2007); and A. Agarwal et al, "Highly ordered Nanostructures for Ultra-Sensitive SERS", in the proceedings of the 14th International Conference on Solid-State Sensors, Actuators and Microsystems, Lyon, France, June 10-14 2007). The micrograph which is Fig. 1 of the latter document shows that the surface in the cavities are each different and within each cavity there are irregularities. In these references pure gold (or silver) is deposited over a semiconductor surface including regularly arranged nanostructures. Note that the SERS substrates disclosed in these references were proposed for a completely different application: analysis of a liquid sample with only a single analyte. The idea was that this single analyte might have a stronger SERS spectrum due to the nanostructures on the surface, although the physical mechanism for this was not entirely understood. It was not then appreciated that these substrates could be useful for liquid samples with multiple analytes. There was no attempt to compare spectra collected at different areas of the SERS substrate: simple spot measurements were made.

- Another way to produce inhomogeneity in the surface of the SERS device is to form different areas of a single SERS substrate with different respective compositions, or alternatively to use multiple SERS devices having different compositions to collectively form the SERS surface. The different compositions will in general have different

adsorption characteristics, so that the analytes will be adsorbed to different relative levels in the different areas. The spatially non-uniform adsorption of molecules means a greater variation in the measured Raman spectra from point-to-point on the SERS surface, and this variation is a crucial pre-requisite for successful BTEM analysis.

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For example, one way to produce inhomogenous composition is to form the SERS surface from an alloy of a first metal (such as gold or silver) having a strong SERS effect with at least one further material (e.g. another metal). The further material may be an other element such as any alkali metal, transition metal, rare earth metal, metalloid or non-metallic element. The further material is typically present in a much lower proportion than the first metal, such as under 10%, more preferably under 7%, and optionally at no more than 5% atomic concentration. In this case, variations in the concentrations of the further material – which arise spontaneously on the microscopic scale – lead to spatial variation of the analyte concentrations on the microscopic scale.

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In a further possibility, the SERS surface may comprise at least one area with a number of components of the alloy greater than two. Specifically if the first metal is alloyed with a number N of further materials which is at least 2 (referred to here an “N-component” alloy, where it is understood that N is at least 2), then the variations are greater. N-component alloys have many different domains on an atomic scale. For example, a 3-component gold alloy (perhaps prepared from three binary alloys containing gold and respectively materials A, B, C in a proportion of say 1%) should have primarily domains of gold, but there will also be domains with isolated A, pairs of A, clusters of A, pairs of A-B, clusters of A-B, pairs A-C, clusters of A-B-C and so forth. In other words, by preparing N-component alloys one can cover a lot of combinations rapidly and hence cover a lot of variations in preferential adsorption of the analyte mixture components. It is believed that N-component gold alloy SERS devices would cover compositions and hence preferential adsorptions that are not well represented by binary gold alloys (or even an “ensemble” of binary alloys, as described in the following paragraph). Signal variation should be enhanced for a set of measurements and subsequent BTEM analysis of this set of measurements should result in facilitated spectral reconstruction / signal-to-noise enhancement.

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Alternatively, instead of relying solely on spontaneous inhomogeneity in a binary alloy or an N-component alloy, the SERS surface may be designed to include a first predetermined area which is a first metal in a pure state (e.g. substantially pure gold or silver) and a second area which is the first metal alloyed with at least one further material (that is, a binary alloy or an N-component alloy). Optionally, there may be a third area which is the first metal alloyed with at least one other material. More generally, there be any number of areas (greater than two), each with a different respective chemical compositions. A system in which there are multiple areas of the SERS surface with different compositions is called here an "ensemble". The areas typically have a size of at least 1 micron.

As noted above, the multiple areas do not have to be distinct parts of a single integral (that is one-piece) SERS substrate. Instead, multiple, separate SERS substrate elements, having a different respective surface compositions, may be arranged (though not necessarily fixed together, or even touching each other) to form collectively a single SERS surface. If so, the adsorbed "layer" is composed of multiple layer portions: one on each of the devices.

In principle, in some embodiments the layer portions need not be formed on all of the SERS substrate elements at the same time. For example, in some embodiments a portion of a liquid sample may be used to form a first layer portion on a first SERS device, a second portion of the liquid sample may be used to form a second layer portion on a second SERS device, etc. Locations in each of the two or more layer portions are then measured to generate respective Raman spectra, which are then combined to form a single dataset which is input to the BTEM algorithm. Note that although it may be preferable to form all the layer portions at the same time (e.g. in the case that the liquid sample is changing with time), this is not absolutely necessary in these embodiments of the invention, e.g. the second layer portion may be formed after the first layer portion has been destroyed.

Note that the variations in the composition of the surface produced by the use of the ensembles and/or the use of a binary alloy or N-component alloy surface, have another effect, besides leading to an inhomogeneity in the distribution of the adsorbed analytes. Specifically, the varying composition may induce electronic changes in the SERS process itself. This, in turn, would lead to an inhomogenous Raman enhancement caused by the SERS effect even if the distribution of the analytes on the SERS surface were uniform. It is expected that the pure component spectra would be substantially the same at each location, but that their ratios in the total Raman spectrum for each location would differ.

The analyte components of a liquid sample may be measured only once by the present method. Alternatively, its analyte components may be measured repeatedly. This is particularly interesting in the case of a reacting liquid, since the progress of the reaction may be tracked.

Optionally, the liquid sample may be static during the measurement. Alternatively, liquid samples may be drawn from a fluid in a reservoir, and onto the SERS substrate as a continuous process. This permits a process of continuous monitoring, referred to here as a “flow-through” mode. This would be useful, for example, for monitoring the fluid, e.g. for a process of quality control. Optionally, the fluid in the reservoir may be undergoing a chemical reaction, for example, and the method can be used to monitor this reaction. Alternatively, the fluid in the reservoir may be not be undergoing a reaction, but may be subject to a risk of impurities being formed. In this case, the invention may be useful for quality control (e.g. if the fluid is water). For example, there may be a process of determining whether the identified analytes comprise one of a number of proscribed materials (e.g. poisonous impurities), and in this case performing an alarm protocol.

Brief description of the figures

An embodiment of the invention will now be described, for the sake of example only, with reference to the following drawings, in which:

Fig. 1 is a flow diagram of an embodiment of the invention;

Fig. 2 is composed of Figs. 2(a) and (b), which are Raman spectra respectively of ibuprofen and caffeine deposited from a water sample onto a surface of an SERS substrate;

Fig. 3 is a visible light image of the surface of a SERS substrate onto which a liquid sample containing ibuprofen and caffeine has been evaporated in a first experiment;

Fig. 4 shows respective Raman spectra for 121 pixels on the SERS substrate of Fig. 3;

Fig. 5 is composed of Figs. 5(a)-5(d), and shows pure component spectra obtained from the spectra of Fig. 4, and the spatial distribution of the components;

Fig. 6 is a comparison of the spectrum of Fig. 5(a) with a reference spectrum;

Fig. 7 is a comparison of the spectrum of Fig. 5(b) with a reference spectrum;

Fig. 8 is composed of Figs. 8(a)-8(f) and shows visual images and Raman spectra for three areas of a SERS substrate in a second experiment;

Fig. 9 shows pure component spectra obtained from the spectra of Fig. 8;

Fig. 10 is composed of Figs. 10(a) and 10(b), and compares of the spectra of Fig. 9 with reference spectra;

Fig. 11 indicates the spatial distribution of the components shown in Fig. 9 in one of the areas of the SERS substrate of Fig. 8;

Fig. 12 shows schematically an apparatus which is an embodiment of the invention;

Fig. 13 is composed of Figs. 13(a)-13(f) and shows visual images and Raman spectra for three areas of a SERS substrate in a third experiment, performed using the apparatus of Fig. 12;

Fig. 14 shows two spectra obtained from the Raman spectra of Fig. 13;

Fig. 15 is composed of Figs. 15(a) and 15(b) and is comparisons of the spectra of Fig. 14 with reference spectra;

Fig. 16 is composed of Figs. 16(a) to 16(c), and indicates the spatial distribution of the components shown in Fig. 14 in one of the areas of the SERS substrate of Fig. 13;

Fig. 17 shows Raman spectra from three areas of a SERS substrate in a fourth experiment;

Fig. 18 shows six spectra obtained from the Raman spectra of Fig. 17;

Fig. 19 is comparisons of the spectra of Fig. 18 with reference spectra;

Fig. 20 is composed of Figs. 20(a) and (b), and compares pure component spectra for two analytes obtained using various different SERS surfaces, and by combining the data from these surfaces;

5 Fig. 21 shows Raman spectra from three areas of a SERS substrate in a fifth experiment;

Fig. 22 shows two spectra obtained from the Raman spectra of Fig. 21;

Fig. 23 is comparisons of the spectra of Fig. 22 with reference spectra; and

10 Fig. 24 is composed of Figs. 24(a) and 24(b), and compares pure component spectra for two analytes obtained using various different SERS surfaces, and by combining the data from these surfaces.

Detailed Description of the embodiments

15 Fig. 1 shows the overall flow diagram of a method which is an embodiment of the invention. In a first step (step 1), a mixture of analytes is combined with a fluid to form a liquid sample. Of course, if the sample to be analysed is already present in liquid form, as in certain of the experiments described below, then this step is not necessary. In a second step (step 2), the liquid sample is used to form a layer on the surface of an
20 SERS surface in which the analytes are present in differing proportions at each of a number of "pixels". In a third step (step 3), Raman spectra are collected in respect of each of the pixels. In a fourth step (step 4), the BTEM method is used to obtain pure component spectra in respect of each of the analytes.

25 In some forms of the embodiment, a fifth step (step 5) is performed, in which the pure component spectra are identified using a library of pure component spectra, or by comparison with a first-principles predicted SERS spectrum of a species believed to be present as an analyte.

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1st experiment

A first experiment to demonstrate the effectiveness of the invention was performed using the analytes caffeine and ibuprofen.

5 Firstly, to obtain pure component reference spectra for these two analytes, caffeine and ibuprofen were individually dissolved in water. A sample of the water was deposited on the SERS substrate described in R.Z. Tan et al "3D arrays of SERS substrate for ultrasensitive molecular detection", Sens. Actuators A: Phys. (2007); and A. Agarwal et al, "Highly ordered Nanostructures for Ultra-Sensitive SERS", in the proceedings of the 14th International Conference on Solid-State Sensors, Actuators and Microsystems, 10 Lyon, France, June 10-14 2007). Then the water was evaporated. The Raman spectrum was measured. Fig. 2(a) shows the pure component reference spectrum obtained for ibuprofen, and Fig. 2(b) shows the corresponding pure component reference spectrum obtained for caffeine.

15 Then a sample of water containing a mixture of caffeine and ibuprofen was deposited on the SERS substrate. A Raman microscope was then used to collect a Raman spectrum from each of a 121 pixels on the substrate (specifically, the area of the substrate used was 200x200 microns, with a step size between pixel measurements of 20 microns) to give corresponding mixture SERS spectra. Fig 3 shows a visible light 20 microscopy image of the SERS substrate after evaporation of the solution. It is clear that some sort of pattern now exists on the surface. Fig 4 shows the corresponding Raman spectra from the 121 pixels. Clearly, the spectra in each pixel are quite different, implying the heterogeneous nature of the deposited layer.

25 A BTEM analysis was performed on the 121 spectra. Two patterns were recovered, as shown in Fig. 5(a) and 5(b). Figs. 5(c) and 5(d) are corresponding plots showing spatial distributions of the corresponding intensities of the respective patterns across the SERS surface, where the intensity is marked by a corresponding level of shading. The right side of Figs. 5(c) and 5(d) shows the key to the levels of shading.

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A comparison of the pure component reference spectra of Figs. 2(a) and 2(b), and the respective BTEM estimates of Figs. 5(a) and (b) respectively, show that there are

similarities between them. Most of the main bands are apparent in both, and it can be seen for example that Fig. 5(a) is much more like the ibuprofen spectrum (Fig. 2(a)) and Fig. 5(b) is much more like the caffeine spectrum (Fig. 2(b)). This is more evident still from Fig. 6 which shows Figs. 2(a) and Fig. 5(a) overlaid, and Fig. 7 which shows Fig. 5 2(b) and Fig. 5(b) overlaid.

It is concluded that the embodiment has succeeded in confirming the identity of the two analytes. In other words, if it had not been known that the two mixed analytes had been ibuprofen and caffeine, the embodiment would have demonstrated that. Note that the 10 BTEM method did not use pre-existing knowledge of the pure component spectra, or even of how many pure component spectra there were. The BTEM analysis exhaustively searches the data until no more patterns can be found.

2. Second experiment

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In a second experiment, 8 gold-based SERS devices were investigated, namely eight SERS substrates in which the SERS surface is respectively composed of:

- (i) Au,
- (ii) an Rh/Au alloy in which Rh is 1% and the remainder Au ("1%Rh/Au"),
- 20 (iii) a Ce/Au alloy in which Ce is 1% and the remainder Au ("1%Ce/Au"),
- (iv) an Mn/Au alloy in which Mn is 1% and the remainder Au ("1%Mn/Au"),
- (v) a P/Au alloy in which P is 0.15% and the remainder Au ("0.15%P/Au"),
- (vi) a Ca/Au alloy in which Ca is 1% and the remainder Au ("1%Ca/Au"),
- (vii) a Ca/Au alloy in which Ca is 2.5% and the remainder Au ("2.5%Ca/Au"),
- 25 and
- (viii) a Ca/Au alloy in which Ca is 5% and the remainder Au ("5%Ca/Au").

Note that these proportions (and indeed the proportions of all the alloys described in this document) are by prepared (nominal) weight. The reason for the selection of materials alloyed with the gold (i.e. P, Ce, Ca, Mn, Rh) was that we wanted to survey a base 30 metal, a precious metal, a lanthanide, a metalloid as dopant, and also to survey various atomic / ionic radii. These eight devices were tested using a liquid sample of methylene blue and rhodamine B in water.

We will firstly report in detail an experiment performed using device (iv), that is 1%Mn/Au, as the SERS substrate. Reference solutions of methylene blue in water and rhodamine B in water were prepared at concentrations of about 0.1 parts-per-million.

5 Also, a mixture sample containing both methylene blue and rhodamine B in water was prepared. A drop of about 2 μL (0.002 cm^3) of each of these three solutions was applied to the 1%Mn/Au SERS device. The water in the drop was allowed to evaporate. The Raman spectra were then measured.

10 The spectra for the two liquid samples containing respectively methylene blue and rhodamine B, are shown by lines 11 and 12 on Figs. 10(a) and 10(b) respectively.

Raman point-by-point mapping was performed on three areas of the SERS substrate which had been exposed to the mixture sample. The mapping size for each area was 80
15 μm by 80 μm . The interval step size for each mapping was 10 μm . Hence, for each of the three areas, 81 Raman mixture spectra were collected, and de-spiked and baseline corrected. Figs. 8(a) to 8(c) are visible microscopy images of the three areas. Figs. 8(d) to 8(f) show the corresponding Raman mixture spectra after de-spiking and baseline
20 correction.

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BTEM deconvolution was then performed to give results shown in Fig. 9.

Five pure component spectra were obtained from the algorithm, as illustrated. The top two pure component spectra were identified as corresponding respectively to the methylene blue and rhodamine B. This was done by comparing the two pure component
25 spectra with the spectra 11, 12 which had been obtained by Raman spectroscopy from the two liquid samples containing only methylene blue and rhodamine B. This comparison is shown in Figs. 10(a) and 10(b), where the top two lines in Fig. 9 are marked as 13, 14. It can be seen that BTEM analysis has successfully deconvoluted the mixture spectra into the underlying pure component spectra. The BTEM estimates of
30 methylene blue and rhodamine B have high similarity to the pure references.

The lower line in Fig. 9 is attributable to a background signal due to the SERS device. The third and fourth lines in Fig. 9 were additional and unexpected pure component spectral estimates. These may involve impurities from the solution preparations, the contact with the air, or even some sort of degradation compounds arising from exposure of the methylene blue / rhodamine B to air while adsorbed on the surface.

Figure 11 illustrates how the embodiment can be used to find the spatial distribution for the components in the portion of the SERS substrate contacted by the mixture sample. The left part of Fig. 11 is the central visual image from the upper row of Fig. 8. The left of the image is the interior of the droplet and last part of the spot to dry. The central part of Fig. 11 is the same as Fig. 9. The right part of Fig. 11 shows, for each pixel of the visual image, the corresponding signal intensity of each of the five components. These were obtained by mapping the pure component spectral estimates back onto the experimental measurements and obtaining the weighting for each component. Note the non-uniform concentrations of the components in the analysis area

Similar experiments were conducted using the other seven gold and gold-alloy SERS substrates, namely those formed using Au, 1%Rh/Au, 1%Ce/Au, 1%Mn/Au, 0.15%P/Au, 1%Ca/Au, 2.5%Ca/Au, and 5%Ca/Au. Again, these assay experiments assays used a liquid sample containing methylene blue and rhodamine B mixtures. In these experiments, pure component spectra for both methylene blue and rhodamine B were recovered – along with some other unknown pure components. All eight devices were found to give acceptable performance, but the 0.15%P/Au device, and all the Ca/Au devices gave relatively poor performance in that they only showed strong signals from either methylene blue or rhodamine B. It may be that in presence of air, the P/Au and Ca/Au devices oxidize or otherwise destroy the analytes. The Mn/Au alloy gave the best performance, and better than a surface formed of Au alone, in that the variations from point to point were greater, and hence the signal-to-noise of the BTEM spectral estimates were better.

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As for the concentration of the dopant material, alloys with very high weight percentage of the dopant will probably not exhibit good performance. Therefore, dilute alloys are

preferable. Concentrations of about 5% and below are believed to be preferable. However, it is quite possible that a very low percentage of the dopant metal might already show significantly different adsorption properties compared to pure gold, and hence be desirable.

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3rd experiment

In the third experiment, the same 8 gold-based SERS devices (i)-(viii) were investigated, namely those having surfaces respectively formed of Au, 1%Rh/Au, 10 1%Ce/Au, 1%Mn/Au, 0.15%P/Au, 1%Ca/Au, 2.5%Ca/Au, 5%Ca/Au. Again, the analytes were methylene blue and rhodamine B in water. However, in contrast to the 2nd experiment, the liquid sample was not evaporated onto the SERS devices. Instead, the experimental arrangement was as illustrated in Fig. 12. A reservoir 121 in the form of a glass flask contained water 122 with a low concentration of methylene blue and 15 rhodamine B. The water 122 was drawn from the reservoir 121 through a conduit 123 by a peristaltic pump 124, and into a Teflon holder 125 containing the SERS chip, before returning to the reservoir 121, so that the flow was cyclic. A Raman microscope 126 was focused on the SERS chip which was mounted in the Teflon holder 125. CaF₂ and ZnSe single crystals were used at various times as windows for the holder 125.

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We will describe in detail such an experiment performed using the SERS substrate (iv), that is Au with 1% Mn as a dopant. The liquid 122 in the reservoir 121 was about 10 ml of water inserted into the reservoir 121 with the pump 124 turned on. Methylene blue was then added to the reservoir 121 to achieve a concentration of about 5ppm, and 25 rhodamine B was added to achieve a concentration of about 50ppm. Raman spectral mapping was performed.

Three different mapping areas of the SERS substrate were used. Each mapping area size was 400 μm by 400 μm . The interval step size for each mapping was 50 μm . Hence, for 30 each mapping, 81 Raman mixture spectra were collected. Figs. 13(a), (b) and (c) show the visible microscopy of the mapping areas, and Figs. 13(d), (e) and (f) show the corresponding Raman mapping data, following de-spiking and baseline correction. As

the visible microscopy shows, the surfaces appear spatially homogeneous. However, the associated sets of Raman spectra show that in fact, there is pixel-to-pixel variation for the signals.

- 5 The collected mixture spectra from three areas, after de-spiking and baseline-correction, were collected together for BTEM analysis. Figure 14 shows the BTEM spectral estimates of the underlying individual component spectra. In contrast to the measurements in the 2nd experiment, there are no additional and unexpected pure component spectral estimates. It appears that in flow-through mode, the multi-
- 10 component system undergoes less or no degradation. This may be due to the avoidance of contact with air etc. Fig. 15 shows how the two pure component spectra of Fig. 14 are identified, by comparison of them with reference spectra obtained from methylene blue and rhodamine B. The two lines of Fig. 14 are marked as lines 21 and 22, and the reference spectra as lines 23 and 24. It can be seen that BTEM analysis has successfully
- 15 deconvoluted the mixture spectra into the underlying pure component spectra. The BTEM estimates of methylene blue and rhodamine B have considerable similarity to the pure references.

- Fig. 16 shows a typical spatial distribution for the components in the mixture analysis.
- 20 Fig. 16(a) is the visible microscopy image Fig. 13(c). Fig. 16(b) the pure component BTEM spectral estimates of Fig. 14, and Fig. 16(c) is the resulting spatial distributions, with the right hand part of the figure being scales indicating the meaning of the shading levels. Fig. 16(c) was obtained by mapping the pure component spectral estimates back onto the experimental measurements and getting the weighting for each component.
- 25 Note the non-uniform concentrations of the components in the analysis area.

- Similar flow-through experiments with methylene blue and rhodamine B mixtures were conducted using the other seven gold and gold-alloy SERS substrates, namely those formed using Au, 1%Rh/Au, 1%Ce/Au, 1%Mn/Au, 0.15%P/Au, 1%Ca/Au,
- 30 2.5%Ca/Au, and 5%Ca/Au. In these experiments, only 2 pure component spectra were recovered – for methylene blue and rhodamine B. Other unknown spectra were not

obtained. The signal to noise level in these experiments was slightly less than in experiments 1 and 2.

There are several possible applications for flow-through SERS analysis combined with BTEM analysis. For example, one application would be the use of a SERS chip and BTEM for an HPLC (high-performance liquid chromatograph). Another would be a detector for use in in-situ liquid phase reaction studies i.e. during syntheses. Another would be as a continuous detector for process lines, such as water purification facilities, beverage plants etc.

In this experiment all the alloys were successful, including the 0.15%P/Au device, and all the Ca/Au devices. Based on the fact that the all of P, Ce, Ca, Mn, Rh were successful as dopant materials, it is believed that most elements would be successful when alloyed with gold (or silver) as the main material of the SERS device. As for the concentration of the dopant material, again alloys with very high weight percentage of the dopant will probably not exhibit good performance. Therefore, dilute alloys are preferable. Concentrations of about 5% and below are believed to be preferable. However, it is quite possible that a very low percentage of the dopant metal might already show significantly different adsorption properties compared to pure gold, and hence be desirable.

4th experiment

The 4th experiment resembled the 1st and 2nd experiments, except that instead of a single SERS substrate, the experiment employed multiple SERS substrates, each having a SERS surface with a different respective alloy composition. We refer to such a combination of different surfaces as an "ensemble". Raman spectra obtained from the set of SERS substrates were combined to give a single dataset which was then input to the BTEM algorithm. The compositions were selected from the following possibilities, corresponding respectively to the eight substrates used in the 2nd experiment: Au, 1% Rh/Au, 1% Ce/Au, 1% Mn/Au, 0.15% P/Au, 1% Ca/Au, 2.5% Ca/Au, 5% Ca/Au. The test mixtures consisted of methylene blue and rhodamine B in water.

Note that the use of multiple SERS substrates is equivalent to forming the ensemble as a single SERS device having many sectors with different respective alloy compositions. Such a device can be formed by simply mounting a mosaic of the individual SERS
5 devices (each having a SERS surface of a single respective composition) on the same holder. Alternatively, such a SERS device could be formed by using a mask to cover a silicon substrate and then depositing a first alloy (e.g. Rh/Au), then removing the mask and depositing a different mask, and depositing a second alloy (e.g. Ca/Au) etc.

10 We will describe in detail one such experiment performed using a SERS substrate with three areas, having respectively the compositions: (i) Au with 1% Mn; (ii) Au with 1% Rh and (iii) Au with 1% Ca. RAMAN spectra are collected at multiple respective locations within each of the areas The total set of SERS spectra are shown in Figure 17. Note that there is a lot of variation in the signals.

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The collected mixture spectra from the ensemble of 3 gold-alloys were collected together for BTEM analysis. Figure 18 shows the BTEM spectral estimates of the underlying individual component spectra. Pure component spectra were obtained for methylene blue and rhodamine. Additionally, four unexpected pure component spectral
20 estimates were obtained, corresponding to unknown impurities. As mentioned above with reference to the 2nd experiment, these may involve impurities from the solution preparations, the contact with the air, or even some sort of degradation compounds arising from exposure of the methylene blue / rhodamine B to air while adsorbed on the surface in assay mode.

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Figure 19 shows a comparison of methylene blue and rhodamine B pure component spectra 31, 32 obtained from BTEM estimates from the ensemble of 3 gold-alloys, and reference spectra 33, 34. The BTEM estimates of methylene blue and rhodamine B have a high degree of similarity to the pure references.

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Figure 20(a) shows the methylene blue spectra obtained by a BTEM analysis of the 1%Rh/Au area only (bottom line), the 1%Mn/Au area only (middle line), and the ensemble of 3 gold-alloys (top line).

5 Figure 20(b) shows the Rhodamine B spectra obtained by a BTEM analysis of the 1% Ca/Au area only (bottom line), the 1%Rh/Au area only (2nd line from the bottom), the 1%Mn/Au area only (3rd line from the bottom), and the ensemble of 3 gold-alloys (top line).

10 As Figure 20 shows, the analysis of the ensemble of 3 gold-alloys provided, in general, better signal-to-noise ratios for the spectral estimates (particularly for Rhodamine B, for which the top line in Fig. 20(b) is considerably less noisy than the other three). The advantages of an ensemble of gold and gold-alloys for mixture analysis is anticipated to become more marked as one goes from 2-solute systems to 3-solute systems and to
15 more complex systems.

5th experiment

The 5th experiment used the flow-through arrangement of the 3rd embodiment, but with
20 the ensembles of the 4th experiment. We give detailed results for an ensemble in which the SERS substrate has three areas with different respective compositions. Again, RAMAN spectra are collected at multiple respective locations within each of the areas. The total set of SERS spectra are shown in Fig. 21. Note that there is a lot of variation in the signals.

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The collected mixture spectra from the ensemble of 3 gold-alloys were collected together for BTEM analysis. Fig. 22 shows the BTEM spectral estimates of the underlying pure component spectra.

30 In these flow-through experiments, no extra unknown spectra were discovered. This contrasts with the assay analysis of the 4th experiment.

Fig. 23 shows the comparison of the methylene blue and rhodamine B pure component spectra 41, 42 (shown in Fig. 22) obtained by BTEM from the ensemble of 3 gold-alloys, with reference spectra 43, 44. The lines 41 and 42 have a high degree of similarity to the lines 43, 44. In fact, lines 41 and 43 are so close as to be almost
5 indistinguishable.

Fig. 24 shows a comparison of BTEM analyses of 1%Mn/Au & 1%Rh/Au & 0.15% P/Au individually and the BTEM analysis from the ensemble of gold-alloys. The BTEM analysis of the ensemble of gold-alloys provided much better signal-to-noise ratios for
10 both of the spectral estimates. In particular, the Rhodamine B is much better resolved, as demonstrated by the portions of the spectra for 1%Mn/Au and 1%Rh/Au shown dashed. The advantages of an ensemble of gold and gold-alloys for mixture analysis is anticipated to become more marked as one goes from 2-solute systems to 3-solute systems and to more complex systems.

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Many variations of the experiments above may be made within the scope of the invention. In particular, the 2nd to 5th experiments were performed with binary gold alloys (i.e. gold plus one other component which is an alloy material), but they can also
20 be performed with N-component gold alloys (with N being an integer greater than 2).

Claims

- 5 1. A method of analyzing a liquid sample comprising a liquid medium and a plurality of distinct analyte components, the method comprising:
- (i) contacting a SERS surface with the liquid sample;
 - (ii) performing Raman spectroscopy at each of a plurality of locations of the SERS surface, to obtain a respective Raman spectrum for each of the plurality of
 - 10 locations of the SERS surface, each of the Raman spectra comprising multiple signal components which are pure component spectra from the respective multiple analyte components, the Raman spectra having different respective ratios of the signal components; and
 - (iii) analyzing the plurality of Raman spectra together by an algorithm which
 - 15 extracts a plurality of component spectra, the component spectra comprising pure component spectra which are respective estimated Raman spectroscopy spectra for the analyte components.
2. A method according to claim 1 in which the algorithm is a blind-source
- 20 separation algorithm which does not employ prior knowledge of the Raman spectrum of any chemical.
3. A method according to claim 2 in which the algorithm is a band target entropy minimization algorithm.
- 25
4. A method according to any preceding claim in which a layer of the liquid sample is formed on the SERS surface, and the analyte components are present in the layer in spatially inhomogeneous proportions.
- 30 5. A method according to any preceding claim further comprising determining the proportion of the signal components at each of the locations using the respective Raman spectrum and the pure analyte component spectra.

6. A method according to any preceding claim in which the SERS surface is spatially inhomogeneous.
- 5 7. A method according to claim 5 in which at least part of the SERS surface is an alloy of a first metal with one or more different elements.
8. A method according to claim 7 in which at least part of the SERS surface is an alloy of the first metal with at least two other elements.
- 10 9. A method according to claim 7 or 8 in which the first metal is Au.
10. A method according to claim 8 in which the alloy is selected from the group consisting of Rh/Au, Ce/Au, Mn/Au, P/Au and Ca/Au.
- 15 11. A method according to claim 7 or 8 in which the first metal is Ag.
12. A method according to any preceding claim in which the SERS surface includes one or more areas with a first chemical composition, and one or more areas with a second, different chemical composition.
- 20 13. A method according to any preceding claim in which in step (i) the liquid sample is deposited on the SERS surface, and the liquid medium is evaporated to deposit the analyte components in said locations in different relative proportions.
- 25 14. A method according to any of claims 1 to 12 in which step (ii) is performed while the analyte components are contained in the liquid medium, and adsorbed on SERS surface.
- 30 15. A method according to claim 14 in which the liquid sample is passed continuously from a reservoir over the SERS surface while the Raman spectra are obtained.

16. A method according to claim 14 or claim 15 in which the fluid in the reservoir is subject to a chemical reaction, and steps (ii) and (iii) are performed at each of a plurality of successive times, whereby signal intensity of at least one said signal component is
5 indicative of the reaction progress.

17. A method according to claim 15 which is a process for controlling the quality of liquid in the reservoir.

10 18. A method according to any preceding claim in which the SERS surface is a surface of a one-part SERS substrate.

19. A method according to any of claims 1 to 17 in which the SERS surface is the combined surfaces of a plurality of separate SERS substrate elements.

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20. An apparatus for analyzing a liquid sample comprising a liquid medium and a plurality of distinct analyte components, the apparatus comprising:

at least one SERS device presenting an inhomogeneous SERS surface, for producing an adsorption layer in a liquid sample located on the SERS surface;

20 a RAMAN spectrometer for collecting Raman spectra at each of a plurality of locations of the adsorption layer, to obtain a respective Raman spectrum for each of the plurality of locations of the adsorbed layer, each of the Raman spectra comprising multiple signal components which are pure component spectra from the respective multiple analyte components; and

25 a processor for analyzing the plurality of Raman spectra together by an algorithm which, if the Raman spectra have different respective ratios of the signal components, extracts a plurality of component spectra, the component spectra comprising pure component spectra which are respective estimated Raman spectroscopy spectra for the analyte components.

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21. An apparatus according to claim 20 further comprising a mechanism for drawing a liquid sample from a reservoir and passing it over the SERS surface.

22. An apparatus according to claim 20 or 21 in which at least part of the SERS surface is an alloy of a first metal with one or more different elements.
- 5 23. An apparatus according to claim 22 in which at least part of the SERS surface is an alloy of a first metal with at least two other elements.
24. An apparatus according to claim 22 or claim 23 in which the first metal is Au.
- 10 25. An apparatus according to claim 24 in which the alloy is selected from the group consisting of Rh/Au, Ce/Au, Mn/Au, P/Au and Ca/Au.
26. An apparatus according to claim 22 or claim 23 in which the first metal is Ag.
- 15 27. An apparatus according to any of claims 20 to 26 in which the surface includes one or more areas with a first chemical composition, and one or more areas with a second, different chemical composition.
28. An apparatus according to any of claims 20 to 27 in which the Raman
20 spectrometer is a spectroscopic microscope.