



# An overview of emerging hyphenated SEM-EDX and Raman spectroscopy systems: Applications in life, environmental and materials sciences



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## ABSTRACT

This review covers the potentials and limitations of novel hyphenated SEM-EDX/Raman spectrometer systems (different set-ups) to typify complex materials from disparate fields, as outlined through case studies in bio- and geomaterials, minerals, forensic science, pharmaceutical materials, and cultural heritage items. Emphasis is placed on analytical advantages, restrictions and challenges that must be faced to optimize analyses and achieve the full capabilities of this emergent analytical tool. Our aim is to promote its use and encourage users to explore new applications on challenging materials, by providing published analytical protocols and guidelines.

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## 1. Introduction

The last two decades have demonstrated that there is great demand, seen to be crucial in various fields of science, for a particular type of physicochemical technique. Specifically, this should

provide profound morphological, chemical and molecular characterization of complex nano- and micro-sized material specimens, whether of purely organic, inorganic or hybrid composition. It must preclude sample preparation -to preserve original structures-, and must be performed in the same region of interest (ROI) via a unique instrument. Since such a task is a substantial analytical challenge, this request has recently driven the hyphenation of scanning electron microscopy-energy dispersive X-ray spectroscopy (SEM-EDX) and Raman spectroscopy (RS) into a single system. The novel

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technique offers new insights to unravel the nature and structure of certain specimens not yet entirely characterized. Its development is the result of the collective efforts of innovative scientists searching for powerful and versatile analytical techniques to tackle holistic characterization of hybrid materials. SEM-EDX and RS are full-grown and robust techniques with numerous proven applications in diverse scientific fields [1,2]. A review of the basic principles of both techniques, and the technical aspects involved in coupling them can be found elsewhere [3].

Combining SEM-EDX and RS results in a potent analytical approach that merges morphological and elemental information provided by SEM-EDX, with molecular, structural and electronic data obtained with RS. The key aspects of the success of this hyphenated system are sample visualization at high spatial resolution and chemical information delivered by SEM-EDX, and identification of polymorphs, allotropes and organic/inorganic components supplied by RS. Most materials characterization requires visual examination of the sample, which usually is done with optical microscopy (OM) and/or SEM. The high spatial resolution and good depth of field typical of SEMs make them an ideal device for a material's first observation. Thus the benefit of performing analyses using a hyphenated SEM-EDX/Raman system in place of using RS furnished with an OM (micro-Raman spectroscopy, MRS hereafter) is that SEMs supply accuracy in visualizing sample features. SEMs overcome the restrictions of MRS, moreover using a field emission (high resolution) SEM (FESEM) concerning image spatial resolution (up to 4 orders of magnitude better than OM) and depth of field and contrast, such that the ability to recognize and analyze a ROI is simpler and more precise than using MRS (MRS resolution is ca. 1–2  $\mu\text{m}$ , it is ca. 3 nm for conventional SEMs and ca. 1 nm for FESEM). The additional capability of SEMs to perform elemental analysis when connected to an X-ray spectrometer (SEM-EDX) increases the versatility of this hyphenated technique.

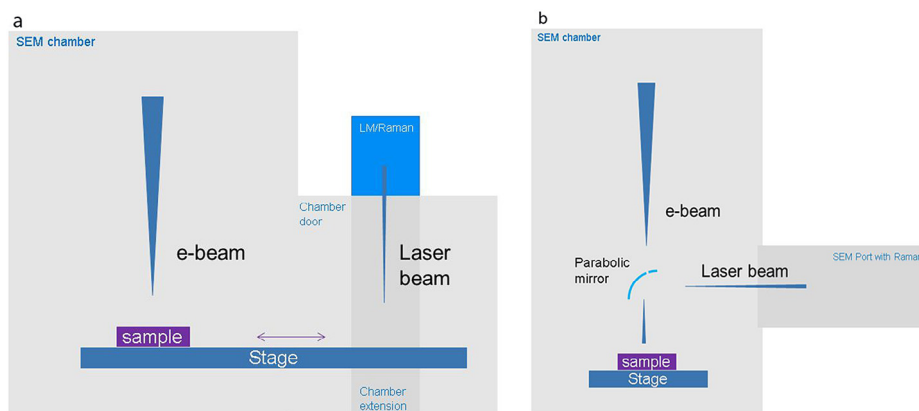
In spite of this, as we will show in the reviewed articles, new practical and technical challenges must be faced when performing coupled SEM-EDX and Raman analyses, since Raman bands are weaker than in MRS, sometimes to the point that Raman signals cannot be obtained. These difficulties and limitations arise from the mutual influence between SEM and RS when connected, as reported in several of the revised papers. The solutions require comprehensive knowledge of factors affecting Raman spectra, expert operators and optimization of analytical procedures -including sample substrate effects [4–7].

## 2. Hyphenated SEM-EDX/Raman spectrometer systems

Different technical set-ups can be implemented to produce unique hyphenated systems. In this section we show the present state of the art in coupling SEMs and Raman spectroscopic techniques. In 2000 the development of such hyphenated systems began with custom-built systems. Once the related technical difficulties in coupling the stand-alone techniques had been solved, hyphenated systems with diverse set-up configurations began to be marketed, although at different times. At present there are four commercial systems available on the market based on two different principles, i.e. correlative microscopy (CM) and *in-situ* SEM-Raman measures using an interface positioned below the SEM's pole piece (Fig. 1).

### 2.1. Correlative SEM -Raman microscopy systems

CM is used to depict the combined information obtained from the same ROI of a sample utilizing at least two different types of microscopy techniques. Correlative SEM-Raman microscopy systems mean that the two systems are “off-axis” indicating that the Raman optics are settled outside the (SEM) electron beam axis. Here the SEM-EDX and Raman data are acquired sequentially after a precisely calibrated movement of the SEM stage. Initially SEM-Raman CM involved time-consuming relocation of a ROI; however at present advanced CM supplies automated procedures to guarantee fast and accurate analysis of a target region. The original non-commercial home-built system of SEM-Raman CM was designed by Aksenov and co-workers in 2000 under the name CRM-SEM (confocal Raman microscope-SEM/EDX) [8,9], later renamed CRSEM (confocal Raman SEM) by Van Apeldoorn et al. [10]. In 2011 *HybriScan Technologies* BV (*HybriScan* company, The Netherlands), together with JEOL and FEI companies as partners, marketed a correlative Raman-SEM microscopy system under the name of *HybriScan Molecular Microscope* (HSCMM), allowing correlated SEM-EDX and MRS analyses of the same object (<http://www.nanounity.com/sem-raman.php>). HSCMM consists of three main modules: a Raman spectrometer, an optical module to SEM and a *HybriScan* pick-up module mounted in the SEM vacuum chamber. After SEM examination of a sample, the SEM stage moves that sample under the lens of the *HybriScan* Raman microscope. A design of this “off-axis” system can be found in [3]. The HSCMM can be integrated with an EDX cryogenic module [11].



**Fig. 1.** Schematic of (a) correlative SEM-Raman microscopy systems showing the Raman optics settled outside the SEM electron beam axis; SEM and Raman data are sequentially acquired after a precisely calibrated movement of the SEM stage; and (b) *In-SEM* Raman systems based on an “on-axis” principle; here simultaneous SEM and Raman measurements are performed on a ROI (Courtesy of S. Freitag, Carl Zeiss Microscopy GmbH).

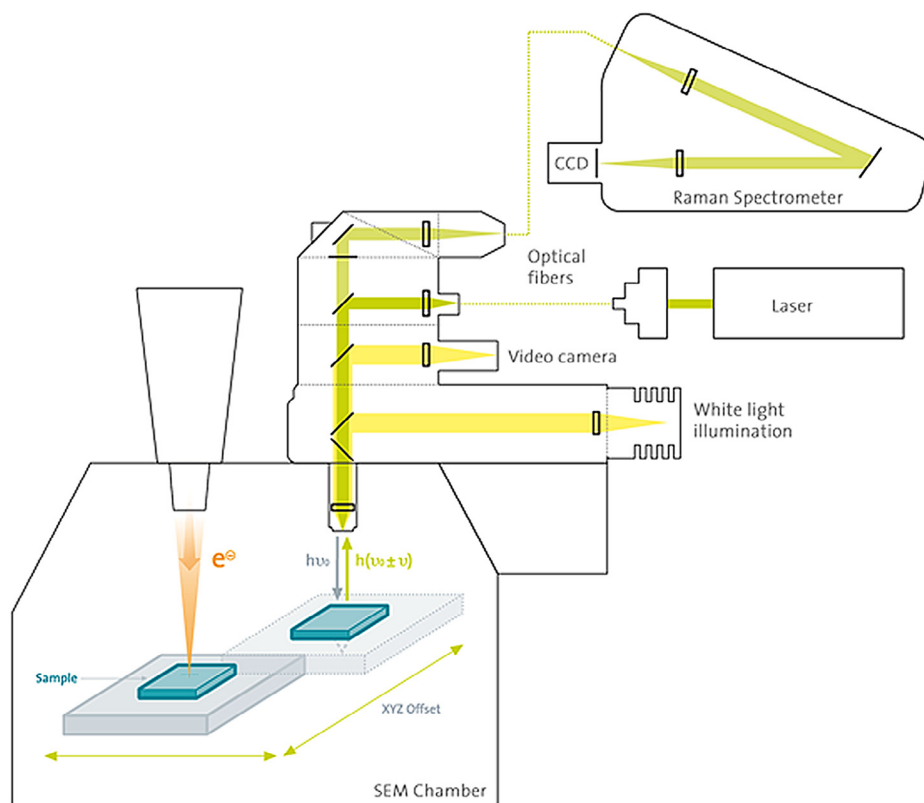


Fig. 2. Design of the correlative confocal Raman Imaging and Scanning Electron (RISE) Microscopy (courtesy of WITec).

In 2014 WITec GmbH company (Ulm, Germany, <http://www.witec.de>), in association with Tescan manufacturer (Brno, s.r.o. Czech Republic, <http://www.tescan.com>), launched the RISE microscope [12]. This is a novel fully-integrated CM technique which combines confocal Raman Imaging and Scanning Electron (RISE) Microscopy (Fig. 2). A precise scan stage automatically transfers the sample inside the microscope's vacuum chamber and repositions it. This is a nonupgradable system that can generate confocal imaging (3-D images) followed by the overlay of SEM and Raman maps (Fig. 3). At present, integration of the Confocal Raman Microscopy (CRM) is also offered by the manufacturer Zeiss (<http://www.zeiss.com>), and is expected for development by other

companies. Five RISE systems have been sold in 2015. Consequently no articles applying this system are yet found in the literature.

## 2.2. In-SEM Raman systems

These systems are based on an “on-axis” principle, meaning that the Raman signal is acquired under the SEM pole piece (Fig. 1a). Hence the Raman system is directly integrated into the SEM. The coupling is made via a retractable arm, equipped with a parabolic mirror inserted into the SEM chamber that can be moved between the pole piece and the sample. Thus simultaneous SEM and Raman measurements are performed on a ROI. Two manufacturers offer

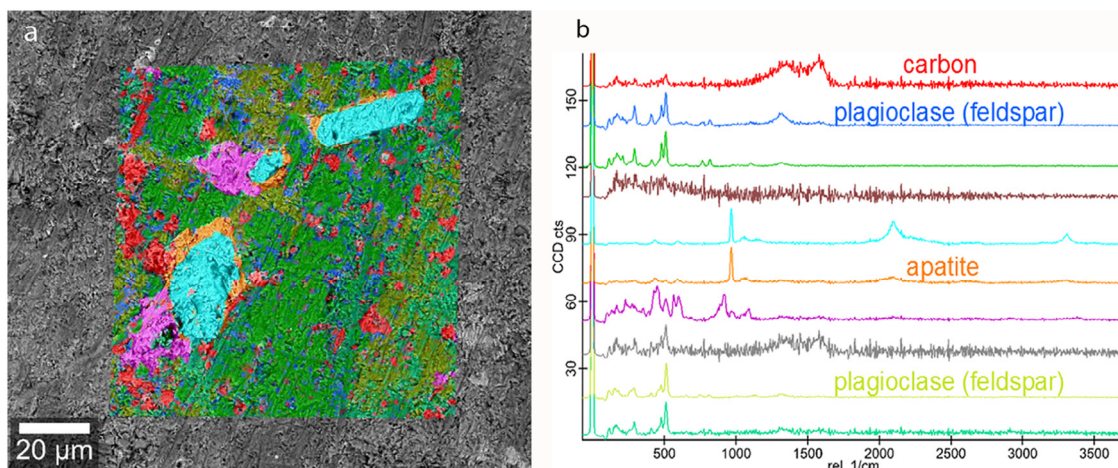
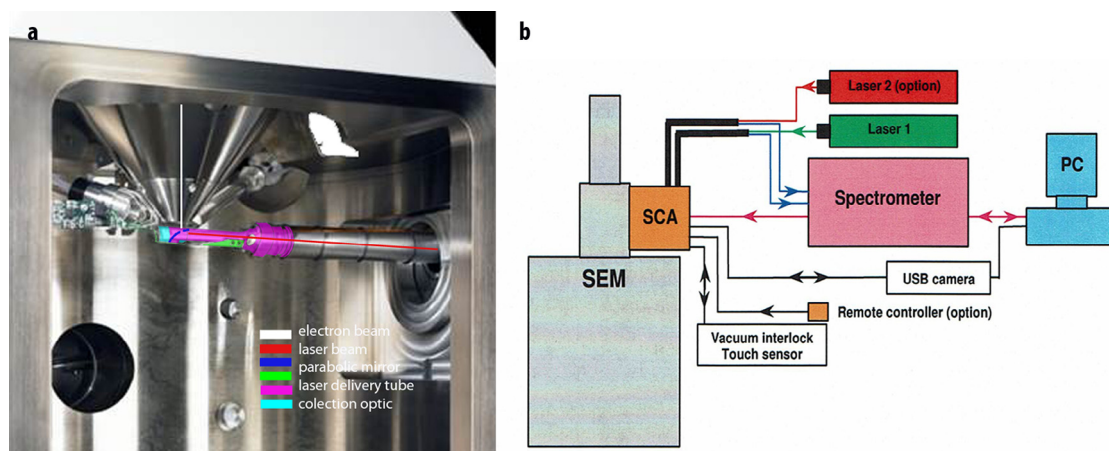


Fig. 3. (a) Image showing overlain secondary electron SEM micrograph (grey) and Raman (color) map. (b) Raman spectra of phases from the left picture [12].



**Fig. 4.** (a) Schematic of the *Renishaw* Structural Chemical Analyzer (SCA) system showing the retractable arm inside the SEM chamber. (b) Architecture of the SCA system (courtesy of *Renishaw*).

in-SEM Raman systems which can be upgraded, *Renishaw* (<http://www.renishaw.com>) and *Horiba* (<http://www.horiba.com>). In 2003 *Renishaw* launched its commercial analytical system called the *Structural Chemical Analyzer* (SCA) which combines Raman spectroscopy (RS) or micro-Raman spectroscopy (MRS) with SEM-EDX (different models). To couple both techniques *Renishaw* uses a parabolic mirror specifically designed for Raman spectroscopy in the SEM. This system introduces the laser light into the SEM focused on the sample (via a 50X objective) and collects the Raman signal through the RS (Fig. 4a). An optical fiber is used to transmit the laser light and the Raman signal between the SCA and RS. The architecture of a SCA system is shown in Fig. 4b and its description can be found elsewhere [3,7]. The *Horiba* system is also coupled with a cathodoluminescence (CL) universal extension (CLUE) which enables acquisition of CL images and Raman mapping. Only one system is available on the market, and publications regarding applications are not available in the open literature.

### 2.3. Historical development

The first description of a device combining a SEM-EDX and a Raman spectrometer was published in 2000 by Aksenov et al. [8]. This non-commercial, home-built system was a CRM coupled to a SEM-EDX named CRM-SEM, as mentioned above. The aim for such a design was to characterize hybrid (organo-mineral) materials, since the combined capacities of both SEM-EDX and RS allowed parallel analyses of inorganic and organic constituents at the same sample spot. In 2002 these authors published several applications of this new hyphenated technique for bioresearch [9]. Simultaneously in 2001 Nelson et al. [4] presented an analytical approach that coupled a *FALCON* Raman Chemical Imaging System with a SEM-EDX, developed by *RJ Lee Group* (*RJ Lee Group, Inc.*, Monroeville, Pennsylvania) and *ChemCon Inc.* (Pittsburgh, PA) respectively. The new method was used to characterize atmospheric particulate matter (PM). In this hyphenated system, samples had to be physically transported from one instrument platform to the other, and particle relocation had to be performed manually. In 2003 *Renishaw* manufacturer (Gloucestershire, UK) marketed its new SCA technique, and published several short papers about applications [13]. For instance, carbon nanotubes were analyzed in cooperation with the Naval Research Laboratory of Washington DC [14], and bacteria were studied working with the University of Manchester (Dept. of Chemistry) [15]. Likewise *Renishaw* analyzed diamond grown around a tungsten wire core [16], polymethylmethacrylate beads in collaboration with *JEOL* manufacturer (MA, USA) and the Application &

Research Center from Tokyo [17], as well as contaminant particles in pharmaceutical products [18].

The first commercial SEM-Raman CM technique was the RISE microscope, which went on the market in 2014 [12]. Since publications showing its use to analyze materials are still lacking, most of the articles reviewed in this paper concern studies performed with different SCA models, apart from those carried out with home-made systems. Irrespective of the hyphenated SEM-Raman systems used to perform analyses, their full capabilities and limitations are constantly examined in nano-sized substances from distinct research fields. The first full paper related with this technique was published in the field of aerosol science by Nelson et al. [4]. The authors applied SEM-Raman CM to characterize atmospheric fine PM. Five years later, several articles appeared in the literature analyzing individual mineral particles of diverse origins. In all these studies, in-SEM Raman analyses were done, and results proved that this hyphenated technique was very effective to characterize such types of airborne PM. Indeed most of published studies have been performed on mineral compounds, such as PM [5,19,20], mineral particles [6,21–24], metal-rich particles [25,26], pigments, ceramics and metals [7,27–30] and U-rich compounds [19,31,32].

Papers addressing biological applications appeared in 2004. Bacterial analyses were done applying an in-SEM Raman system combined with a surface-enhanced Raman scattering method (SERS method) to increase the bacteria's weak Raman signal by colloidal Ag-labeling [15,33,34]. In 2005 the structure and chemical composition of bone extracellular matrices were examined using an "off-axis" CRSEM system [10]. In 2011 carotene compounds in food matrices were investigated by utilizing cryo SEM-Raman CM [35]. Pioneering in applying the hyphenated SEM-Raman technique to the field of cultural heritage was the University of Bologna (Italy), which in 2008 started publishing articles using the SCA system on pigments [27], ceramics [28] and bronzes [29]. Papers analyzing historical paintings performed by Spanish Universities appeared beginning in 2012 [7,30]. In 2015 in situ SEM-Raman investigations were performed on advanced strategic high tech materials such as graphene (different states) [36].

### 3. Applications, difficulties and solutions

Coupled SEM-Raman analyses cover numerous fields of science; however few articles have been published to date. This is due to the limited number of research centres, laboratories, and private institutions that are capable of applying this hyphenated tech-

nique at this time. Thus in this review the revised case studies are sparse.

### 3.1. Life science

The first published papers using the hyphenated SEM-Raman technique was in the field of life science; particularly an SCA system was applied to tackle bacteria discrimination [33,34]. RS is an effective technique for the rapid identification of bacteria and fungi. However they show weak Raman signals that fortunately can be enhanced by SERS methods. The SERS process relies mainly on either the adsorption or proximity of an analyte to a metal substrate. Such substrates include roughened metal surfaces, colloidal solutions, or roughened electrodes [37]. Jarvis et al. showed the reproducible collection of information-rich bacterial SERS spectra using an SCA system [33]. Replicate Raman spectra were acquired from Gram-positive and from Gram-negative bacteria samples prepared for SERS using  $\text{NaBH}_4$ -reduced Ag colloid. The SERS method reduced the spectral acquisition times. Additionally, Principal Component Analysis (PCA) was applied on their SERS fingerprints allowing the grouping bacteria according to their nature. The benefit of using the SCA system here relied on the imaging power provided by the SEM to identify the ROI which aided the acquisition of Raman spectra. In a later paper [34] these authors applied a similar analytical approach to discriminate by SERS fingerprints and principal components-discriminant function analysis (PC-DFA): 1) Gram-negative vs Gram-positive bacteria; 2) closely related Gram-positive bacteria; and 3) bacterial spore biomarkers (genus *Bacillus*). The detection of spores is vital in food processing laboratories, and in detecting its potential use as a biological warfare agent. Up until this study, Gram-positive species were hard to distinguish using genetic methods.

In 2005 Van Alpendoorn et al. performed a study in tissue engineering science applying a CRSEM system [10]. In this field, inorganic and organic components are commonly combined (e.g. mineral phases and collagen in bone matrix) and need to be analyzed at high-resolution in the most non-invasive way to maintain their original structure. This was the first paper dealing with the challenging task of analyzing hybrid compounds. The authors investigated an in vitro formed bone extracellular matrix (ECM) produced by osteoprogenitor cells obtained from rats on Ti alloy plates. Results revealed that ECM was made of collagen type I fibres and carbonated apatite-like calcium phosphate ( $\text{CO}_3$ -AP). Prior to do the study, authors' first goal was to assure the efficacy of this CM system in terms of ROI relocation, for which they used 2–10  $\mu\text{m}$  polystyrene beads. Another problem encountered was the difficulty to interpret Raman images with increased complexity in ECM surface topography.

In 2011 two papers dealing with the analysis of biological specimens were published. Both studies were conducted using cryo preparation methods with the aim of preserving the original microstructure and composition of those biological samples [11,35]. The investigation of Hazekamp et al. [11] was conducted on sucrose and polystyrene beads ( $\varnothing = 2$ –10  $\mu\text{m}$ ) with an EMRAM instrument. This was a home-built CM system that combined an MRS and an environmental SEM (ESEM) designed at the University of Twente (The Netherlands), to which a customized cryo preparation and transfer system was added. Samples were placed on TEM grids to precisely relocate a ROI. Analyses were done at ambient, cryogenic temperatures and during a heating ramp. Well-resolved Raman bands were obtained at cryogenic conditions, still preserving the samples' native structure. Though very promising, the authors mentioned troubles related with the sample cryo preparation, since occasionally heat damage by the laser can affect the stability of the frozen-hydrated samples [11].

In the paper of Lopez-Sanchez et al. a variable pressure (VP) FESEM-SCA (MRS) with an attached cryo system was used to lo-

calize lycopene and  $\beta$ -carotene compounds in tomato- and carrot-based emulsions containing oil [35]. The aim was to shed light on the effect of processing conditions on the distribution of lipophilic micronutrients in plant-based food systems with potential health benefits. Samples (cross-sections without metal coating) were sublimated to remove accumulated frost and cooled for Raman analyses. The authors faced two main problems in this study: i) ice crystal artefacts appeared on the sample surface due to the freezing process, and ii) correlation of the FESEM and MRS measures on the same ROI, helped with the use of 10  $\mu\text{m}$  beads. In spite of this, good Raman spectra were obtained. Results revealed that amorphous  $\beta$ -carotene was present in the carrot cells and the oil droplets in the food matrix, whilst amorphous lycopene was found in both the tomato cell material and the oil droplets.

### 3.2. Materials science

This section deals with research carried out on micro-sized mineral particles of diverse origin, such as PM, toxic element-rich minerals and geomaterials. Usually SEM-EDX, MRS, EPMA (electron probe micro-analysis) and XRD (X-ray diffraction) are used to characterize these materials [38,39]. However more sensitive analytical techniques are needed, and in this regard SEM-Raman systems fulfill this requirement. As mentioned above, most of published studies have been done on mineral compounds, which are discussed below, categorized into distinct applications areas.

#### 3.2.1. Particulate matter

PM comprises micrometric solid and liquid matter (dust, soot, smoke, smog, ...) suspended in the Earth's atmosphere. Their full physicochemical characterization is crucial due to their impact on our climate, environment, human health and artworks, among others fields [38,40]. Often their nature is quite complex since they can be a mixture of organic and inorganic substances such as minerals and metals. A great number of studies regarding PM related risky effects have been published in the last decades [40,41]. Of particular interest for human health are  $\text{PM}_{2.5}$  ( $\varnothing < 2.5 \mu\text{m}$ ) and  $\text{PM}_{10}$  ( $\varnothing < 10 \mu\text{m}$ ), which are able to enter into the human respiratory tract.

In 2001 Nelson et al. [4] reported the first study performed in atmospheric PM samples ( $\text{PM}_{2.5}$ ) using a home-built SEM-Raman CM system. The authors tested diverse filter substrates (teflon, polycarbonate, quartz) to be used with both SEM-EDX and RS. The best filter was polycarbonate due to its flatness and because its Raman bands do not coincide with those commonly found in PM. However polycarbonate was not resistant to laser-induced thermal expansion. The main challenges faced during this work were: i) time-consuming manual particle relocation (impossible in some cases), ii) sample movement mostly during Raman analyses, and iii) electron and laser beam damage.

Identification of U compounds and related trace impurities in airborne PM is a demanded and challenging task. The information gained is crucial in relation to nuclear safeguards and accidents, actions performed inside inspected nuclear facilities, or clandestine activities. At present, MRS allows an excellent structural and molecular characterization of U compounds at good resolution ( $\varnothing < 10 \mu\text{m}$ ). However analytical problems arise from fluorescence and laser-induced heating effects. In addition, recognition is difficult due to the complex U speciation (U-O system) and the small particle size ( $\varnothing < 5 \mu\text{m}$ ). To overcome these limitations in the last years few authors have explored the use of coupled SEM-Raman analyses to better typify U-rich particles.

In 2006 Stefaniak et al. [19] analyzed real life U-rich particles with a SCA (MRS) system. The authors showed the challenges faced and experience gained performing the analyses. Again the successful relocation of particles was the most difficult task. The

repositioning of the small ( $\varnothing < 100 \mu\text{m}$ ) U-rich particles was helped using TEM Cu grids. Nonetheless often particles could not be relocated, which was linked to the sample preparation that should facilitate separate particles. To this end three different procedures were tested. U-rich particles were suspended in either water or hexane solution and deposited on Ag foil. Since both solvents may change some properties of the particles, alternatively these were also placed directly on adhesive C discs used for SEM-EDX. Samples were first analyzed with SEM-EDX and then with MRS. Results showed the complex nature of the U-rich samples, where  $\text{PbMoO}_4$ ,  $\text{UO}_2$  and  $(\text{Na,Ca,U})_2(\text{Nb,Ta,Ti})_2\text{O}_6(\text{OH,F})$  were identified among other minerals.

Worobiec et al. in 2010 analyzed airborne PM ( $\varnothing = 5\text{--}100 \mu\text{m}$ ) samples using the SCA technique and stand-alone MRS for comparative purposes [5]. This was the first paper illustrating the optimization of the experimental parameters associated with the SCA system. The authors indicated that to ensure reliable data acquisition and interpretation, the SCA analytical approach needed to be optimized in the following aspects: particle relocation, sequential data acquisition, beam damage, carbon deposition, sample environment (vacuum vs ambient conditions), and type of sample substrate. For atmospheric PM the correct choice of sample collection surface played a key role in the acquisition of credible data. The best option was to use flat, non-aged Ag foils. The additional advantage of using Ag foils is the SERS effect they produced. Particle relocation was solved using a TEM reference grid avoiding particle overloading. As inferred from the revised literature, the adopted solution for a particle or ROI relocation depends on features such as composition, size, and isolation, among others [7]. It was found that Raman intensities recorded under vacuum mode were lower than those acquired in ambient air [5]. Loss of Raman signal intensity in an SCA is critical since Raman active bands can be masked in micro-sized particles and hybrid compounds [7]. The authors also warned about carbon deposition on samples' surface, suggesting to acquire X-ray maps at the end of the analytical procedure to minimize its effect.

Since 2013 papers reporting applications of SEM-Raman analyses are more profuse, and specifically tackle the analysis of risky mineral particles. Goienaga et al. [25] proved that the SCA technique combined with chemical equilibrium models was crucial to unravel the links between atmospheric PM and human health. The authors studied samples collected from an abandoned Zn–Pb mine (blende-galena Mine), that included dust-type PM from soils without plant cover and on lichen surfaces. Thermodynamic simulations were applied to selected minerals identified in the PM, under the chemical conditions of the mammal lungs. The aim was to predict which minerals could be retained in the alveoli or pass into the bloodstream. First SEM images were acquired to recognize particle morphology and size ( $\varnothing < 75 \mu\text{m}$ ). These images were analyzed with an image-processing tool to deduce particle size distribution. Circa 50% were classified as PM<sub>2.5</sub> and PM<sub>1</sub> that can reach the human alveoli or enter into the bloodstream respectively. Afterward, EDX single-point and maps analyses were performed, followed by Raman spectra. First SEM images were acquired to recognize particle morphology, followed by EDX single point, maps analyses and finally Raman spectra. Plattnerite ( $\text{PbO}_2$ ) and carbonates bearing Ca, Zn, Mg, Pb or Fe were found in the soil samples. The SCA analyses were easier to do in the soil samples (inorganic matrix) than in the PM-rich lichen samples (organic matrix) due to lower fluorescence [25]. In this latter X-ray maps helped to recognize mineral species (Zn-rich silicates) not identified with RS. The best Raman spectra were acquired from minerals with higher degrees of crystallinity, and from samples with simpler composition. Results revealed that plattnerite may dissolve and pass into the bloodstream while galena ( $\text{PbS}$ ) can be retained in the alveoli. Also discovered was the effect of the mining pollution ca. 1 km from the mine entrance.

Pointurier and Marie [31] applied a SCA system (MRS) to analyze different produced U oxide particles ( $\varnothing < 10 \mu\text{m}$ ). These were sampled with sticky carbon tapes (SCTs), mounted on a carbon disk, or collected with cotton cloths and deposited on glassy graphite disks (GGDs). The aim was to check the effect of the sample substrate in the SCA analytical response. SCA results were also compared with stand-alone MRS. The reported SCA difficulties were: i) relocation of an ROI with accuracy better than  $1 \mu\text{m}$ . (to this end they used landmarks and TEM grids), ii) signal loss compared to the MRS signal, and iii) the sensibility of U compounds to high laser beam intensities which results in their chemical alteration. Moreover it was found that the actual transmission of the filters was different from the theoretic values, as in [7,22]. U particles with sizes from 1 to  $2 \mu\text{m}$  were successfully analyzed with SCA, whereas MRS only could analyze particles larger than  $5 \mu\text{m}$ . Analyses were greatly influenced by the type of sample substrate. Thus Raman spectra were of better quality for U particles deposited on SCTs. However GGDs were preferable since particle identification was easier and they were suitable to analyze U isotopes.

In 2014 Stefaniak et al. published a study of  $\text{UO}_2\text{F}_2$  reference particles [32].  $\text{UO}_2\text{F}_2$  may deposit as a solid particulate compound on the ground from accidental releases of  $\text{UF}_6$  (handled in enrichment or conversion plants). The authors analyzed the molecular forms of three types of  $\text{UO}_2\text{F}_2$  particles deposited on different substrates (rough and smooth polished and non-polished graphite disks). The study was performed using a FESEM-SCA in high vacuum, and results were compared to MRS analyses in ambient air. The SCA analytical procedure was first to perform an automatic scan of samples to discriminate U particles. Afterwards X-ray microanalyses followed by MRS spectra were obtained. This study also addressed the analytical challenges faced, which mainly were: i) relocation of an ROI for particles smaller than  $2 \mu\text{m}$ , and ii) damage of  $\text{UO}_2\text{F}_2$  by both electron beam and laser beam. Under the electron beam the physical structure of the U particles changed (while the chemical composition remained unaltered) making them unsuitable for Raman analyses. Also  $\text{UO}_2\text{F}_2$  particles exposed to laser beam in vacuum were damaged with a total loss of fluorine followed by conversion to  $\text{U}_3\text{O}_8$ . This study revealed that U-rich particles down to  $700 \text{nm}$  deposited on a smooth polished graphite substrate were successfully identified in vacuum.

### 3.2.2. Geomaterials

Heavy mineral sand (HMS) deposits comprise diverse minerals which are source of materials such as Zr, Ti, Th or W that may form ore deposits, as well as precious metals or gemstones. In 2006 Stefaniak et al. [19] analyzed zircon ( $\text{ZrSiO}_4$ ) and rutile ( $\text{TiO}_2$ ) sands with a SCA (MRS) system. Relocating the isolated, large sized ( $\varnothing = 150\text{--}200 \mu\text{m}$ ) and regular-shaped sand grains was relatively easy. The analytical sequence was to perform MRS analyses first and then coat the sand grains with C for SEM-EDX analyses. While elemental information was key for a correct interpretation of the Raman spectra, MRS allowed detection of entrapped Hf in the zircon-rich particles.

A year later, Potgieter-Vermaak [21] showed the unique fingerprinting ability of the SCA system to typify HMS compared with previously used analytical techniques, such as OM, XRD or X-ray fluorescence (XRF). The author proved the benefit of combining MRS data with X-ray maps for fast, *in situ* estimation of the efficacy of the ore separation process in the HMS. SEM X-ray maps found that Ti-rich particles were associated with O and Zr, and that Zr-rich grains were related with Si and O. This revealed that the separation process was not 100% successful. MRS allowed identification of the two  $\text{TiO}_2$  polymorphs, i.e. anatase and rutile.

In 2011 Worobiec et al. studied haematite ( $\text{Fe}_2\text{O}_3$ ) particles using the SCA technique and stand-alone MRS [20]. The type and control of PM damage under vacuum conditions in SEM-Raman analyses

are more complicated and less understood than using MRS. In the case of Fe-rich particles, although they are resistant to the electron beam, they are laser-beam-sensitive. The authors discussed factors that could cause Fe-rich particle damage, i.e. laser power and excitation, atmosphere of analyses, particle structure and collection substrates (Ag and Al foils, and silicon wafer) [20]. Results revealed that under vacuum mode melting of haematite and recrystallization as magnetite ( $\text{Fe}_3\text{O}_4$ ) occurred with the 785 nm laser, while melting and morphological damage were detected for the 514 nm laser. In this regard, the structure of the particle agglomerates influenced their stability to the laser beam. Thus in vacuum mode isolated particles melted while loosely powdered structures were more resistant. Damage was more evident on the Si wafer substrate. However, since damage effects were unpredictable, the authors resolved that more research is needed to define factors that influence SEM-Raman analyses.

In recent times there are other materials causing environmental and health problems that have attracted the attention of scientists. Among these are the mineral species within the alunite family. This is partly due to their ability to incorporate heavy (toxic) metals and rare earth elements (REE) into their structure. In addition recently the mineral alunite has been identified on Mars, suggesting the presence of aqueous components [42]. Their general formula is  $\text{AB}_3(\text{XO}_4)(\text{OH}_6)$ , where A = Na, U, K, Ag, NH, Pb, Ca, Ba, Sr, light REE, B = Al, Fe, Cu, Zn and X = S, P or As. These minerals form complex solid solution series that make their characterization quite challenging. Likewise aluminum-phosphate-sulfate minerals (APS) of the alunite family display complex chemical zoning which together with their occurrence as clusters of small crystals ( $\varnothing < 10 \mu\text{m}$ ) make them ideal specimens to be studied with hyphenated SEM-Raman systems.

In 2013 Maubec et al. [24] using a FESEM-SCA identified for the first time a micrometric APS with  $\text{PO}_4/\text{SO}_4$  ratio of 1.5/0.5. Polished thin sections of these samples (without carbon coated) were analyzed at low vacuum mode. APS were very sensitive to the electron beam since alkali ion migration took place during analyses. To check the effect of sulfate anion substitution by phosphate anions on the APS structure, other APS minerals with different  $\text{PO}_4/\text{SO}_4$  ratios were analyzed. Raman spectra for all APS samples had bands with similar positions, though with different relative intensities in the  $\text{AP}_{1.5}\text{S}_{0.5}$  specimen. This was attributed to changes in its structure due to a sulfate anion substitution by phosphate anions. It was concluded that the phosphate increment caused an enlargement in  $I(\text{PO}_4)/I(\text{SO}_4)$  intensity ratios. Hence the authors proposed to use this correlation to identify the sulfate and phosphate contents in APS minerals.

Another study dealing with hazardous materials was published by Gómez-Nubla et al. in 2013 [26]. These authors analyzed slags using an analytical strategy that included the SCA technique. The goal was to identify the composition of slag and the weathering products formed by superficial reactions with the atmosphere. Slag comprises heavy metals that can cause environmental and human health problems. This scenario has impelled the search for alternatives to reuse this industrial waste. Slag cannot be used directly; it must be treated to be stabilized. The authors studied slags with and without treatment, from the steel industry and from civil construction works, to find dangerous substances [26]. The SCA analyses (high vacuum mode) were restricted to study compounds at trace levels. The chosen analytical strategy was to obtain SEM images to select target areas followed by EDX mappings and in-SEM Raman analyses. Here no problems of ROI relocation were addressed, due to the big size of the analyzed particles, ranging between  $250 \mu\text{m}$  and  $2 \text{mm}$ . Likewise analyses were easy to perform since neither thermal decomposition nor problems with Raman fluorescence were observed (slag is purely inorganic). Results revealed trace amounts of toxic Cr-rich minerals in samples from the steel industry. Instead

toxic minerals were not found in stabilized slag used in civil construction works.

In 2014 Wille et al. [3,6] published two related papers concerning coupled SEM-Raman analyses. One comprised a clear summary of the history, background and principles of the SEM-EDX and RS techniques, and discussed the need to hyphenate both [3]. The different analytical systems available in the market were also shown. Additionally, the authors reported some case studies where this tool was applied, as well as some investigation on geomaterials performed in their laboratory. A large part of this paper addressed SEM-related phenomena that influence Raman spectra carried out in hyphenated SEM-Raman systems, such as carbon deposition, SEM low vacuum conditions (LVC), working distance (WD), resolution, and cathodoluminescence (CL) for the first time. Some of these topics were also tackled in [6].

Wille et al. conducted their study using a FESEM-SCA under LVC. Some results were compared to those acquired using an MRS. A loss of Raman intensity in the SCA was found compared to signals from MRS, which was attributed to the SCA parabolic mirror, the focal length, and the Raman scattering efficiency of the opaque mineral studied. The authors also warned that CL and carbon deposition on the sample surface could prevent correct Raman spectra interpretation. While CL has a wavelength in the same region where Raman scattering takes place, the key Raman bands for silicates, carbonates, phosphates and sulfates coincide with the main carbon bands. To show the benefits of the hyphenated SEM-Raman technique, the authors analyzed the zonation and twinning of a cassiterite ( $\text{SnO}_2$ ), and the nature of a “milky pearl” biocrystal. Here the  $\text{CaCO}_3$  polymorphs aragonite and vaterite were discerned. However results of the organic matrix present in the pearl structure were not shown. Also fragments of red and black prehistoric cave-art pigments from wall paintings were studied. Pyrolusite ( $\text{MnO}_2$ ) and cryptomelane ( $(\text{K,Mn})_8\text{O}_{16}$ ) were identified in black particles, and Ba substitution of K in these latter. Nonetheless the type of clays and carbonates were not fully characterized. Indeed, this paper only showed results of mineral phases, which suggest problems unravelling the organic composition of the studied hybrid samples.

### 3.2.3. Forensic and other toxic materials

In 2009 Otieno-Alegro [22] analyzed gunshot residues, explosive mixtures and paint flakes from crime and accident scenes using an SCA system. Coupled SEM-Raman analyses are particularly suitable for criminalistics since they allow materials characterization in a non-destructive and non-invasive way without sample preparation. The author addressed limitations of the SCA system and suggested precautions to guarantee data quality. For example, one can use a marked grid to ensure exact ROI relocation, and shut off the electron beam to reduce sample beam bombardment. This last step may result in sample degradation and excessive carbon layer deposition. The author used the SEM imaging capability to rapidly identify target particles, and then collected the Raman data [22]. In the paints MRS identified rutile, calcite ( $\text{CaCO}_3$ ) and barite ( $\text{BaSO}_4$ ), though the organic matter remained unknown due to the weak Raman signals. In the explosives  $\text{KClO}_3$ , S, TNT and Tetryl were found. Here analyses were easier due to the unique inorganic nature of the detached  $50 \mu\text{m}$  sized particles.

In 2013 Ghosal and Wagner [23] tackled the benefits of using ESEM-confocal MRS CM to non-destructively analyze toxic flame retardants (FRs) in house dust samples and TV casing and furniture items. FRs are compounds (minerals, organohalogen or organophosphorus mixtures) added to manufactured materials to reduce or avoid the threats associated with fire. Many FRs and related degradation compounds cause cancer and other health effects. Moreover some of their weathering products are the primary toxic agents, such as dioxins or polybrominated diphenyl ethers (PBDEs). In this study, samples deposited on carbon tab

substrates were first analyzed with SEM (BSE and EDX analyses) to find areas containing Br and/or Sb followed by MRS. The authors did not mention problems of particle relocation, likely due to their distinct morphology and dimension ( $\varnothing \sim 100 \mu\text{m}$ ). However unique physical markings in the particles and SEM Cu grids were used to aid particle relocation. Results revealed randomly distributed deca- and penta-BDE,  $\text{Sb}_2\text{O}_3$  inclusions, plastic remains and other inorganic components in the FRs. The organic compounds were identified without difficulty possibly due to: i) their nature, ii) setting as discrete fragments in the inorganic-rich matrix, and, iii) no resin embedding of the samples. The finding of coexisting plastic fragments and deca-BDE in dust particles indicated their mechanical transfer into the environment via abrasion of consumer products.

### 3.3. Heritage science

Presently the tendency in Heritage Science (HS, i.e. conservation science in cultural heritage) is to apply an array of complementary analytical techniques to analyze inorganic/organic components and crystalline/amorphous phases from art/archaeological items, their alteration products and restoration materials. Hyphenated SEM-Raman systems open a frontier for a conclusive material characterization of such items as shown below.

The first SEM-Raman studies performed in HS were conducted at the University of Bologna (Italy). Analyses were done using a VP-SEM-SCA (MRS) system. The first published paper appeared in 2008 and dealt with the study of “green earths pigments” [27]. The green color is due to the existence of green Fe-rich minerals belonging to the group of clay micas (glaucanite or celadonite), and less commonly to the group of smectites, chlorites or serpentines. Their mineralogical characterization is highly challenging due to their heterogeneity in the paint layer and small crystal size. Thus, the mineral species rarely have been specified in HS studies, although they are crucial to unravel the artwork provenance [43]. Ospitali et al. [27] analyzed pure glaucanite, celadonite and ferroceldonite from diverse countries, others minerals used as “green earths”, fragments from Roman *frescoes* and artistic commercial pigments labelled “green earths”. It should be said that organic binders were not present in the historic samples since calcium carbonate is the binder in mural *frescoes*. This somehow facilitated the Raman identification of the crystalline specimens, since as proven in [7] organic binders complicate the analysis of mineral pigments due to fluorescence problems. Results showed that though Raman spectra of glaucanite and celadonite were very similar, some spectral differences helped to discern between them, as well as to distinguishing them from other “green earth minerals”. In the *fresco* samples glaucanite and celadonite were identified. Only in some cases were the artistic commercial pigments really based on celadonite/glaucanite, since they often contained mixtures of different minerals, impurities and even synthetic dyes. The authors warned about pigments damage when using intense laser power.

The next paper appeared in 2010 and addressed the analysis of Medieval and Renaissance Italian ceramics [28]. Characterization of ceramics, including body and surface decoration (glaze and lustre), is a wide field of research in HS reporting on technological, artistic and historical aspects [44]. MRS has proven to be a powerful tool to this end [45]. Hence, RS coupled to SEM-EDX offers new options to attain more comprehensive knowledge about: i) minor phases and few micrometers phases which could give better insights about ceramics dating and technology, and ii) ceramic pigments composed either of crystalline minerals or chromophore ions dispersed in a glassy matrix. The multi-technique study of Bersani et al. [28], including SCA analyses, fully described ancient Italian ceramics. The SCA was solely used to analyze the glazes and the paintings materials. Here MRS analyses were performed before SEM-EDX analyses.

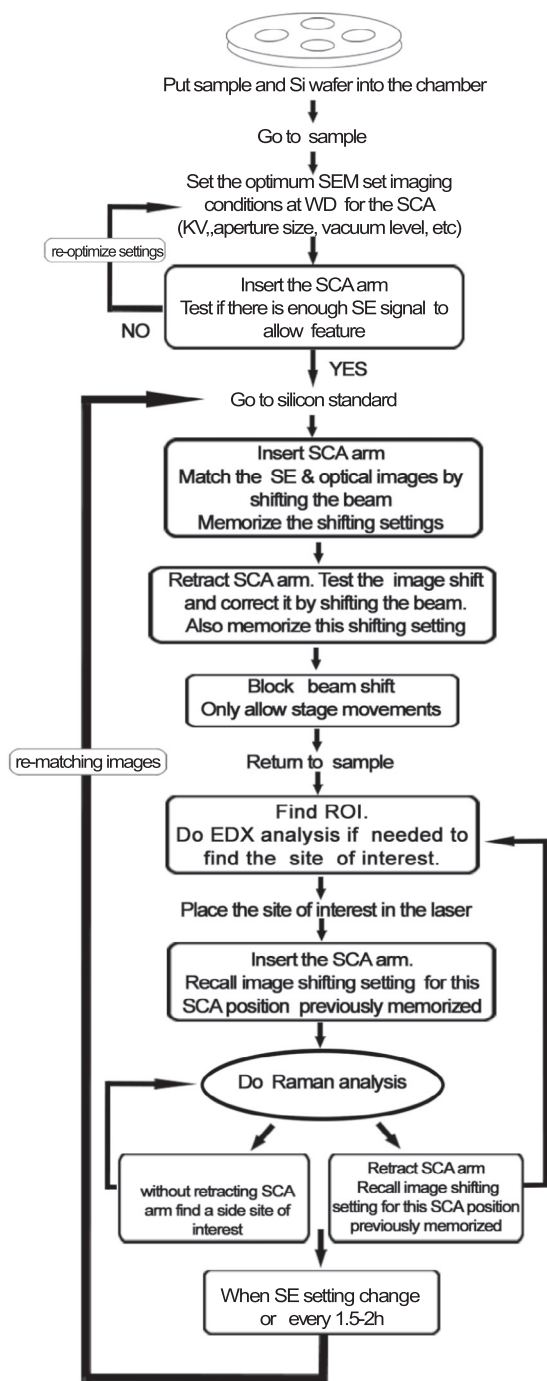
The authors found  $\text{TiO}_2$  anatase in the 17<sup>th</sup> century white glazes. Anatase was used as white pigment (*titanium white*) starting in 1916; however it can be found as an impurity in clays. Thanks to the SCA analyses it was verified that the white glazes were made of white clay used as *ingobbio* (clay covering) and Ti, which was responsible for the Raman spectrum similar to that of pure anatase. On the other hand, Co and Cu identified with SEM-EDX suggested that the colors were attained by dispersion of the chromophore ions in the glassy matrix, since no Raman bands typical of blue and green compounds were acquired. Likewise unresolved Mn phases using MRS were cited from the black areas of glazes.

The third paper published by Ospitali et al. [29] tried to discern crystalline and nano-sized Sn dioxides in bronze patinas from buried archaeological items, and bronze samples exposed to outdoor conditions and an acid rain run-off accelerated ageing test. Bronze corrosion mechanisms that originate patinas are a major issue in HS since the characterization of corroded patinas is crucial to choose the most suitable conservation strategy. As an alloying element, Sn plays a key role in the formation and stability of these patinas. However, typification of Sn-based corrosion products is quite challenging and the research community has been seeking new analytical approaches. Ospitali et al. proved that SCA analyses of bronze patinas facilitate a more precise explanation of their Raman spectra [29]. However problems were not absent during analyses due to: i) the low Raman scattering power, the uncertain structure, and the low crystallinity degree of the Sn-based compounds -generally in amorphous states-, and, ii) Raman bands of Sn oxides overlapping those of Cu oxides. In this study, patinas (chip samples and polished cross-sections) were first examined with SEM to gain morphological information, and then X-ray maps were acquired to discern the Sn distribution used to guide Raman analyses. Results revealed that  $\text{SnO}_2$  was found in patinas from all bronzes, and so the corrosion mechanisms were similar in all environments. The SCA technique helped to demonstrate that bronze corrosion is a decuprification phenomenon, where Sn and Fe contributed to substitution effects in the patinas [29].

In 2012 a paper was published dealing with the *in situ* study of 16<sup>th</sup> century wall paintings from two Spanish churches, their original support, decay products and restoration materials [30]. The authors applied an SCA system as a complementary technique to portable instruments. Only three micro-samples (polished thin sections) were examined with the SCA to know the distribution and composition of the wall paintings and their support (i.e. *intonaco*). Results revealed the infeasibility of obtaining Raman spectra from binders and some pigments when both were closely mixed due to low Raman scattering or high fluorescence. Consequently, it was via *in situ* Diffuse Reflectance FTIR (DRIFT) that the egg tempera technique could be recognized. On the other hand, though in the green areas Co and Cl were detected with the SCA, Raman spectra were not obtained neither from the original green pigment nor from the alteration products. Instead the purely inorganic (calcite, gypsum and anhydrite) *intonaco* samples were easily identified. Results revealed that gypsum ( $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ ) dehydration into anhydrite ( $\text{CaSO}_4$ ) in the *intonaco* was responsible for wall painting detachments.

The application of coupled SEM-Raman analyses in the heritage painting field remained incompletely explored until 2015 with the paper of Guerra and Cardell [7]. The authors analyzed with a VP-FESEM-SCA loose pigments (chip samples), and paint cross sections and metallic-rich samples prepared as polished thin sections. The aim was to identify organic and inorganic components concurrent in a specimen. New analytical challenges were met analyzing multilayered complex hybrid paintings, not yet reported. Hence, the authors indicated the need to design *ad hoc* analytical protocols depending on the studied specimen, and the type of hyphenated SEM-Raman system.





**Fig. 5.** Flow chart of the proposed VP-FESEM-SCA optimized analytical procedure [7].

As mentioned, relocation of an ROI with sub-micrometric precision is vital to properly characterize challenging specimens, such as closely mixed pigments ( $\varnothing < 10 \mu\text{m}$ ) and binders in paint layers, often resin-embedded. Guerra and Cardell [7] designed a strategy to correlate the illumination systems of the FESEM and the RS to assure exact alignment between the laser and electron beams, even when the ROI is not identified via the SCA's video source. They were the first authors to outline an optimized analytical procedure to work with a hyphenated SEM-Raman system (Fig. 5).

This paper also tackled for the first time other issues, among which the following should be highlighted [7]. In-SEM Raman signal intensities were comparable when working at different vacuum

modes, irrespective of the type of laser used. These intensities were lower than those acquired with MRS, which were assigned to factors not yet reported: i) imprecise launching of the laser into the optical fibre, ii) unsuitable fibre core diameter, iii) filter contamination, and iv) incorrect filter setting with respect to the optical fibre head. This last aspect can vary the nominal filter values, as the authors already confirmed in their system. This study also proved that: i) carbon deposition is substrate dependent, ii) minor differences in SEM WD affect Raman intensities, iii) Raman signals increase with slight over focus, and iv) crystals with less volume give worse Raman spectra and are more damaged by the lasers [7]. The authors indicated that samples were more prone to burning in SCA systems than in MRS due to the smaller size of the analyzed ROI and the unreleased heat due to the lack of atmosphere. A SERS study was also conducted on these samples using gold nanoparticles and SEM Al stubs with roughened surfaces as SERS substances. Results revealed that gold over-sputtering hindered Raman signals, and that no SERS effect was achieved from paint cross-sections. Consequently, the authors could not identify organic binders, as in [3,6,22,30], and verified that getting good Raman spectra from specific pigments was also difficult.

#### 4. Concluding remarks and research needs

The development of the hyphenated SEM-Raman technique began in 2000 with the use of home-built CM systems. In 2003 the first commercial system was launched to the market. Over the last two years there has been a re-emergence of this hyphenated technique prompted by significant technical advances in instrumentation and software, and scientists' demand for a new potent analytical tool. Thus, at present new commercial SEM-Raman systems are available; consequently literature on their applications are still lacking. Irrespective of the specific hyphenated system, the main pitfalls addressed by users and manufacturers are noted below.

The accurate relocation of an ROI is a pending issue. In the RISE system this is a question of sample repositioning linked to the mechanical reproducibility and exactness of stage movement. Indeed, CM is based on instrumentation technology and software intended to enhance automatic matching of instruments and images. By contrast, in the SCA system this is a problem related to matching the electron and optical images, so the ROI relocation depends largely on the operator expertise. To solve this drawback, a solution would be to integrate standard software that automatically corrects the SEM and Raman image shifting.

Laser- and Raman-induced sample damage is a real problem when using this technique. It depends largely on the nature and morphology of specimens, electron beam exposure, type of laser wavelength excitation, laser power and laser exposure time. Moreover in hyphenated SEM-Raman systems, heat and electric charges cannot be evacuated from the sample surface due to the absence of atmosphere. This, together with the micro-size of the analyzed features engenders physicochemical modifications and even burning of samples. Integrated cryo-SEM technology or cold fingers in the hyphenated systems would resolve this pitfall. A collateral effect of reducing laser power and exposition times on studied features is the diminution or even loss of Raman signals. The development and implementation of intelligent algorithms to clean Raman spectra will enable the obtention of high-quality Raman spectra. Consequently, identification of organic/inorganic components concurrent in hybrid samples could be possible. On the other hand, since reliable Raman data are compromised by carbon deposition at the sample surface due to electron beam exposure, instruments should be furnished with plasma cleaning devices for the removal of hydrocarbons.

Each hyphenated system displays benefits, drawbacks and applications, which vary with configuration, the nature of studied specimen and the research aims. For instance, CM systems offer

brilliant imaging capabilities, including confocal imaging and 3-D image generation. Thus *a priori* CM systems are recommended for studying large sample areas and seeking relations among phases. Instead resolution and ROI relocation are better in in-SEM Raman systems, specifically the SCA system. Hence the SCA is ideal for analyzing micrometric particles, geomaterials and heterogeneous samples, in spite of their weak image skill that should be improved in the future.

Prior to applying a specific system, users must be aware of these issues, and then select the most suitable hyphenated system for their investigations. Systems should be viewed as complementary rather than exclusive, and none can be considered superior in general. In any case, success depends on well-trained personnel and a carefully planned analytical strategy. This requires prior knowledge of the sample, and research on sample preparation strategies, including sample substrates, resins and SERS methods. Dedicated companies are now very active in marketing SEM-Raman systems. We expect that this hyphenated technique will become a mainstream analytical tool in diverse fields of sciences in the near future.

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