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Flow-through microdispenser for interfacing μ-HPLC to Raman and mid-IR spectroscopic detection

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Abstract

A flow-through microdispenser has been coupled to a micro HPLC separation system and used as a solvent elimination interface for Fourier transform infrared (FTIR) and Raman spectroscopic detection of the separated compounds. Using the microdispenser picoliter sized droplets can be generated and deposited on an appropriate target placed on a computerized *x*, *y*-stage. Evaporation of volatile solvent and buffer is rapid and allows analysis of the obtained dry deposits by various techniques. Due to the destruction free character of Raman and FTIR spectroscopy they can be applied sequentially to interrogate the same deposit. In the reported application five phenolic acids typically present in wine have been separated on a C-18 column technique using a mixture of water, methanol and acetic acid as mobile phase. For spectrum acquisition infrared and Raman microscopes have been used. The spectra recorded from the dried deposits of the separated compounds agreed well with the reference spectra of corresponding components.

Keywords: Raman microspectrometry; FTIR microscopy; HPLC; Flow through microdispenser

1. Introduction

In infrared and Raman spectroscopy important instrumental developments continue to be made especially when considering the analysis of micro-samples. In case the sample can be properly presented to the detector often surprisingly minute amounts of material is sufficient for obtaining a mid-infrared or Raman spectrum with good signal-to-noise ratio. Single bacterial cells [1], organic monolayers [2] or a few femtograms of almost any material are generally enough for obtaining a meaningful spectrum [3].

Development of different detectors to be coupled to modern separation systems is an ongoing field of research. For analyte identification modern spectroscopic techniques that provide a full spectrum are required. Among these Raman and mid-infrared spectroscopy are of special interest due to the molecular specific fingerprint that they provide. Furthermore, their non-destructive character allows their use in sequence as well as coupling with other more sensitive detection schemes. Raman and infrared spectroscopy provide complementary, structural information that can be used for quantitative as well as qualitative analysis. Whereas application of mid-IR spectroscopic detection in liquid chromatography is made difficult by strong absorption of the solvent, in particular of water, Raman spectroscopy is less affected by solvent bands. However, direct application of Raman spectrometry for on-line detection in solution is made difficult by the low concentration sensitivity of this technique [4]. A Raman measurement of the separated analytes in solution has been achieved by diverting the flow to an off-line sampling cell. In doing so long integration times could be applied which allowed for improved sensitivity [5]. Another

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interesting approach is the use of a Raman waveguide detector, which also enables important improvement in sensitivity [6]. To overcome the lack in sensitivity, a more sensitive variant, resonance Raman spectroscopy may be considered for certain applications [7–9]. Better sensitivity and selectivity can be also achieved by applying surface enhanced Raman scattering spectroscopy (SERS) [10,11]. Surface enhanced resonance Raman scattering technique (SERRS) was also tested as off-line detection method in HPLC for even more sensitive Raman measurements [12]. Both techniques are, however, not appropriate for routine applications due to limited stability and reproducibility of the required substrates for surface enhancement. Furthermore, reproducible placement of the analytes eluting from the chromatographic column on the substrates presents an additional problem.

Application of infrared detection to liquid chromatography can be done on-line in a flow-through infrared cell or off-line applying solvent elimination techniques with subsequent condensed-phase analysis. In on-line flow-through cells short optical paths in the low micrometer range must be applied due to infrared absorption of the mobile phase. In case of isocratic separations spectral subtraction of the mobile phase absorption can be performed to derive the mid-IR spectrum of the separated analytes [13–16]. However, the accessible spectral range is limited due to strong solvent absorptions, which practically obscure the corresponding spectral regions. In order to overcome the problem of mobile phase absorption, a lot of research efforts have been devoted to solvent elimination approaches. Among various methods, the use of heated gas nebulizer [17], the use of concentric flow nebulizer [18–20], and ultrasonic [21] or electrospray nebulization [22] have been applied for the elimination of solvents. Some authors have also employed a particle beam [23–26] or a thermospray interface [27–29]. Advantages of solvent elimination are seen in the access to the full mid-infrared spectrum, the potential application of spectral libraries and increased sensitivity as compared to on-line detection using flow cells.

A common characteristic of these solvent elimination techniques is that they generate a mist of tiny liquid droplets in a rather uncontrolled way. The generated mist is partially or fully dried, condensed and deposited on a given target. Achievement of small analyte deposits is of utmost relevance for sensitive infrared and Raman detection. The reported diameters of the so achieved deposits measure a few hundreds of micrometers depending on the technique used. When considering a reasonable sample spot of a mid IR microscope being 50 μm and the required sample spot of a modern confocal Raman microscope of only 1 μm in diameter these deposits are not optimal.

A different promising strategy to achieve small deposits is to use a flow through microdispenser which is capable of producing 50 pl sized microdroplets on demand. These droplets, when deposited directly on a target, give rise to dried deposits with diameters of a few tens of micrometers [30,31]. The microdispensers work stable and are an interesting high precession tool for delivering minute amounts of liquid sample in a controlled and reproducible way. Therefore, they have already found application in different hyphenated systems such as LC–MALDI-MS [32–34] or LC-FTIR systems [35] among others [36,37].

In liquid chromatography there is a clear trend toward miniaturized separation systems such as capillary and nano-HPLC systems. This development is of importance for infrared and Raman detection because an important increase in sensitivity of infrared and Raman detection is possible when moving from normal bore HPLC columns to the miniaturized versions. The reason for this is the experimental difficulty to recover the full amount of a separated analyte dispersed in an HPLC peak together with solvent removal and concentration of the analyte on a small spot for interrogation with an infrared or Raman microscope. As a consequence in conventional sized liquid separation systems the vast majority of available sample is left unused. Efficient recovery of the separated analyte is, however, made easier in case reduced peak volumes are produced by the chromatographic system.

In this contribution a flow-through dispenser has been successfully tested as an interface between micro-bore HPLC and Raman as well as mid-infrared spectroscopic detection on the example of the analysis of phenolic acids.

Wine polyphenols is an interesting and complex group of substances significantly contributing to the sensory properties of wines. Due to their diversity in composition as well as degree of polymerisation the polyphenols are difficult to separate and characterize. Therefore current analytical techniques are focussing on lower molecular weight components such as monomeric phenolic acids. Due to their physico-chemical characteristics, analysis of monomeric phenolic compounds is usually carried out using high-performance liquid chromatography with reversed-phase C-18 columns. In this work we have focused on five phenolic acids (gallic acid, 3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, vanilic acid and syringic acid) from wine water fraction [38] for method development. The eluents described in literature for separation of these compounds comprised mainly methanol–water mixtures which have been applied in gradient modes together with a constant amount of acetic acid [39–41] formic acid [42], perchloric acid [43,44] or phosphoric acid [45]. An acetonitrile–water gradient with constant amount of acetic acid [46] was also used. For the optimum detection of all compounds of interest wavelengths 256 or 280 nm were usually chosen.

2. Experimental

2.1. Solvent elimination interface based on a flow-through microdispenser

A schematic of the used microdispenser is given in Fig. 1. The piezoceramic element of the dispenser was driven by a dc power supply (Manson NP-9615) together

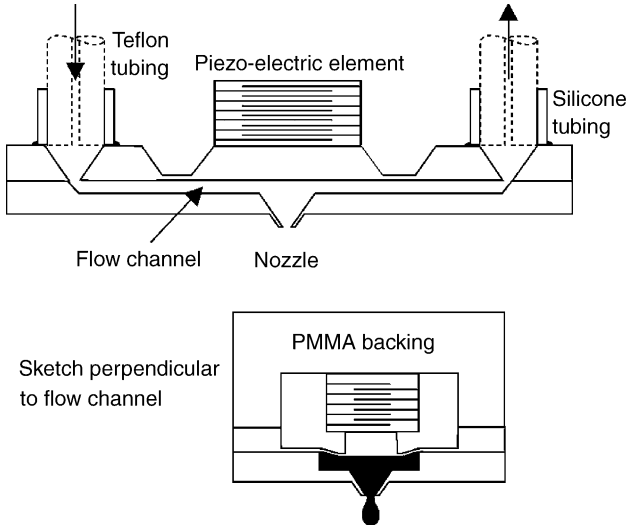

Fig. 1. The principle design of the flow-through microdispenser. Bottom: electrically induced deformation of piezo-element causes droplet ejection.

with a computer controlled arbitrary waveform generator (Agilent 33120A, Agilent Technologies, Palo Alto, CA) which provided and electronic pulse with defined amplitude (15 V), rise (5 μs), width (490 μs), and decay time (680 μs). To enable lateral location of the deposits on the CaF_2 (38 mm × 19 mm × 2 mm) target a computer controlled *x*, *y*-stage (THK, Compact Linear Axis) with step sizes of 5 μm and maximum distance of 90 mm × 40 mm was implemented in the dispensing unit. All the computer controlled components of the microdispensing unit and the sequential injection (SI) system described below were operated with the help of an in-house-written MS Visual Basic 6.0 (Microsoft) based software program (Sagittarius, Version 3.0.25) working under Windows NT operating system.

2.2. Sequential injection and HPLC equipment

For performance assessment of the solvent elimination unit, a sequential injection system was set up. The SI system featured a Cavro XP 3000 syringe pump (Cavro Scientific Instruments, Sunnyvale, CA) equipped with a 1000 μl syringe and a Cavro XL Series 6 port selection valve. The PTFE tubing (i.d. 750 μm) and fittings of the flow system were purchased from Global FIA Inc. (Gig Harbor, WA). In all experiments based on the sequential injection system a flow rate of 50 μl/min was used. The HPLC set up consisted of a Merck/Hitachi L-7100 pump with a Rheodyne 7725 injection valve featuring a 12.5 μl sample loop and a 150 mm × 2.1 mm, 3 μm Nucleosil 100 RP C-18 separation column from VDS Optilab (Berlin, Germany). The column was kept at room temperature and the experiments were performed with 0.15 ml/min eluent flow rate. The eluent consisted of 15% methanol in a 1% acetic acid water solution. After leaving the column the effluent was split using a high-pressure micro-splitter valve from Supelco (Bellefonte, USA). Part of the effluent, with the flow rate equal 20 μl/min, was directed to the microdispenser, the rest went to a CE UV–vis detector (Isco, Lincoln, NE) set at 280 nm. An ELDS Pro 1.0 laboratory data system (Chromatography Data systems, Kungshög, Sweden) was used to register the chromatograms. The UV detection window was made from an untreated fused silica capillary (i.d. 150 μm, o.d. 375 μm, Composite Metal Services Ltd., Hallow, UK) by burning away a small piece of surrounding polyamide coating on the capillary. The capillary was fixed to HPLC PEEK tubings (i.d. 0.13 mm, o.d. 0.16 cm) with an in-house made connection.

2.3. Measurement of Raman and mid-infrared spectra

All Raman spectra of deposits and acids' standards were acquired with confocal Raman microscope (LabRaman HR, Jobin Yvon Ltd., Bensheim, Germany) using a 633 nm laser line and a charge coupled device (CCD) detector with 1024 × 256 pixels. A grating with 600 grooves/mm, a confocal aperture of 500 μm, an entrance slit of 100 μm and an objective lens with 100 times magnification were selected for the experiments. Under these experimental conditions Raman spectra of a spot size of approximately 1 μm in diameter were obtained. Spectra were recorded with data acquisition time ranging from 10 to 60 s.

FTIR spectra were obtained in transmission using a home made IR microscope equipped with a liquid nitrogen cooled mercury cadmium telluride (MCT) detector and attached to a IFS 55 spectrometer (Bruker Optics, Ettlingen, Germany). The spectra were recorded by co addition of 100 scans with a spectral resolution of 8 cm^{-1} at a mirror velocity of 20 kHz HeNe frequency. Using an aperture the IR beam was narrowed down to 50 μm in diameter in order to match the average size of the deposits. For each spectrum a new background spectrum was collected in close vicinity to the deposit in order to keep background instabilities due to surface irregularities of the CaF_2 support at minimum. For on-line transmission measurements a 25 μm flow cell with CaF_2 windows was used.

Spectra of standards of the acids were recorded as KBr pellets on a Vector 22 FTIR spectrometer (Bruker Optics, Ettlingen, Germany) equipped with DTGS detector. They were recorded by co addition of 100 scans with a spectral resolution of 8 cm^{-1} collected at a mirror velocity of 10 kHz HeNe frequency. Each KBr pellet was prepared by mixing of 1 mg substance with 200 mg of KBr.

3. Reagents

The standards used were: *p*-hydroxybenzoic acid (99%) from Aldrich (Steinheim, Germany), gallic acid (purum ACS; ≥98%), 3,4-dihydroxybenzoic acid (purum; ≥97%), vanilic acid (purum; ≥97%) and syringic acid (purum; ≥97%) from Fluka (Buchs, Switzerland). Water for HPLC and methanol for HPLC from Fluka (Buchs, Switzerland)

and acetic acid from Riedel de Haën (Seelze, Germany) were used for the separation procedure and for all standard preparations.

4. Results and discussion

4.1. Test and optimisation of the solvent elimination interface

To evaluate optimal working conditions for the microdispenser as the central element of the solvent elimination interface, the influence of the dispensing frequency and speed of the *x*, *y*-stage on the droplet formation and deposition was studied. For this purpose the dispenser was fed with an analyte solution (1000 ppm vanilic acid in 15% MeOH/H_2O with 1% acetic acid) by the sequential injection system at a flow rate of 50 μl/min. The *x*, *y*-stage was set at translation speed equal to 100 μm/min and the dispenser tested at 5, 10 and 20 Hz dispensing frequency. In these conditions, circular deposit droplets with repeatable shape and size with average diameter of 40, 50 and 70 μm were obtained as a result of the codeposition of 15, 30 and 60 droplets of ca. 60 pl, respectively. The dispensing frequency of 10 Hz was chosen, since it assured formation of deposits with the closer size and diameter to the diameter of the IR beam in the infrared microscope, what assured higher sensitivity and repeatability of the IR detection. Additionally, with higher dispensing frequency, more analyte is transferred to the plate than with the lower one, which is again the reason for better sensitivity of the method. For dispensing frequencies higher than 10 Hz, big deposit droplets with long evaporation times were obtained, resulting in an unequal distribution of the analyte within the droplet since most of it crystallized at the edges. Unequal distribution of the analyte within the droplet results in poorer sensitivity and reproducibility of the method, since for the same analyte concentration in the dispensed solution, the heterogeneity of the deposit provided different spectrum intensities depending on the sampled area.

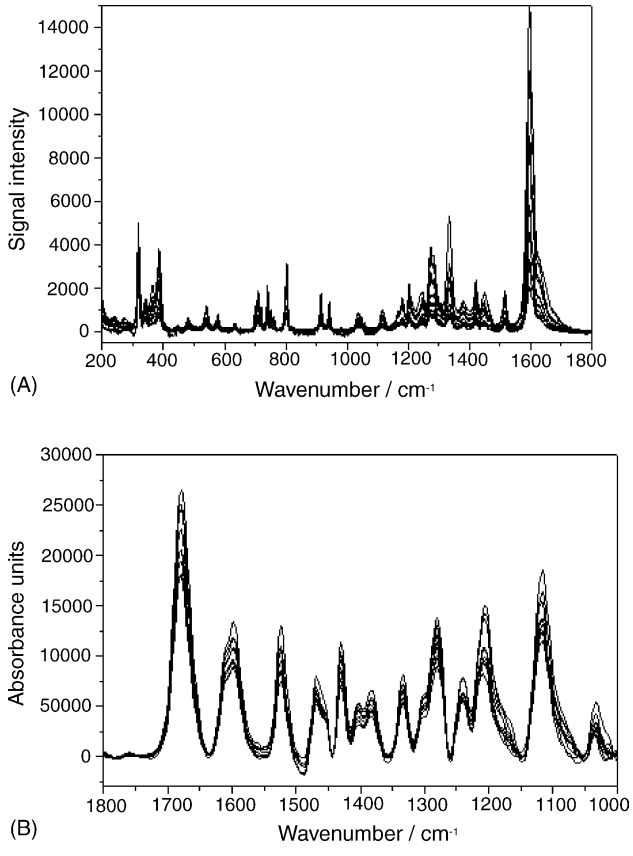

Fig. 2. Reproducibility test for the dispensing procedure done in SIA system for 1000 ppm solution of 3,4-dihydroxybenzoic acid; A—IR measurement, B—Raman measurement.

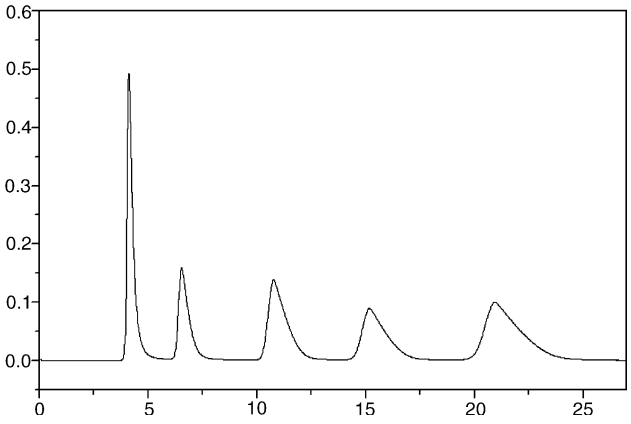

Fig. 3. Chromatogram of 2000 ppm mixture of standards of phenolic acids; flow = 0.15 ml/min, UV = 280 nm, separation done in room temperature in 15% MeOH in water plus 1% acetic acid; 1—gallic acid; 2—3,4-dihydroxybenzoic acid; 3—*p*-hydroxybenzoic acid; 4—vanilic acid; 5—syringic acid.

Once optimised the dispensing frequency at 10 Hz, different translation speeds were tested for the *x*, *y*-stage: 500, 200 and 100 μm/min with the latter being the slowest possible one considering the available instrumentation. As expected, decreasing the translation speed resulted in droplet deposits

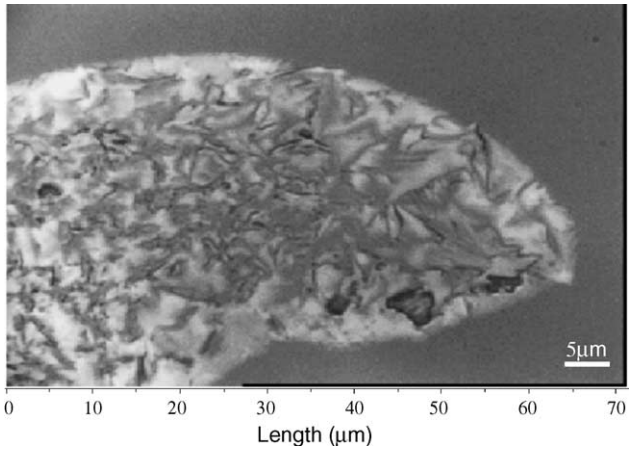

Fig. 4. Deposit of the 3,4-dihydroxybenzoic acid; photo made with the Raman-microscope camera with the 100 times magnification.

of slightly increasing size (20, 35 and 50 μm, respectively) and smaller distances between deposits. Moving speed of *x*, *y*-stage equal 100 μm/min was chosen as these deposits were best fitting the size of the IR beam at the sampling spot.

To check the reproducibility and most suitable detection method for the deposition procedure, 1000 ppm vanilic acid solution (in 15% MeOH/H_2O with 1% acetic acid) was deposited continuously with the 10 Hz frequency over a CaF_2 substrate moving with the speed equal 100 μm/min. All signals obtained from the droplets had the same spectral features (Fig. 2). Relative standard deviation of the obtained signals calculated for ten different deposits was approximately 18.8% for IR detection measured at 1524 cm^{-1} and 51.4% for Raman detection measured at 803 cm^{-1}. Better signal reproducibility was thus obtained for IR detection, probably due to the fact that the IR beam size is close to the deposit dimensions and what is measured is in fact a kind of average from all droplets points. In opposite, Raman spectrum is taken from one point with the diameter of approximately 2 μm, which is quite small compared to the size of the whole droplet (approximately 50 μm) and hence obtained signal is influenced by the lack of homogeneity in analyte concentration within the droplet. That is why μ-IR detection seems to be recommended for semi-quantitative measurements. On the other hand, higher signals were obtained for Raman detection, setting it as a choice for qualitative analysis of diluted samples. This phenomenon can be explained by the high intensity of the laser beam inducing the signal in the μ-Raman measurement.

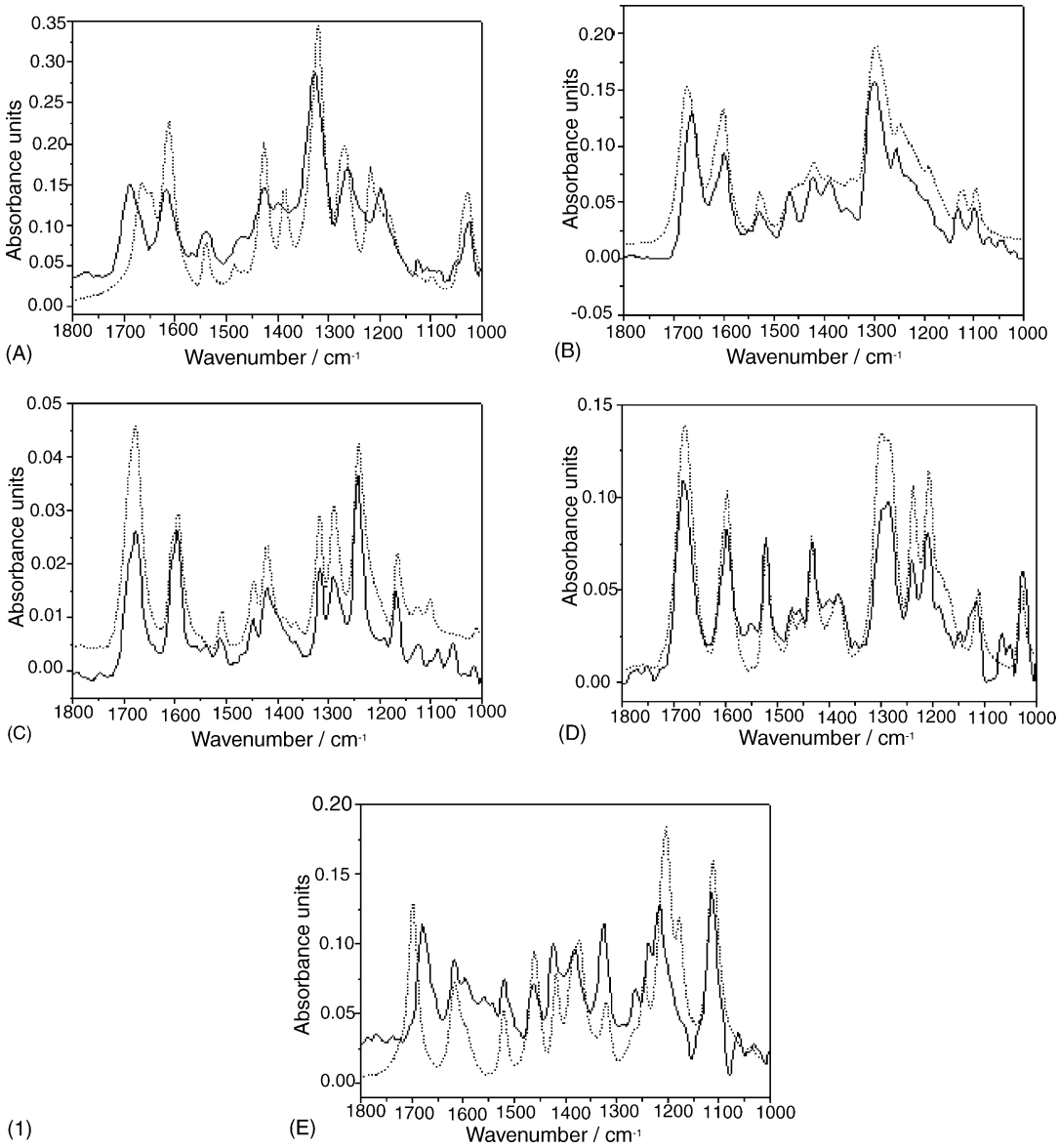

Fig. 5. Infrared (1) and Raman (2) spectra of phenolic acids' deposits (black line) and their standards (dash line); A—gallic acid; B—dihydroxybenzoic acid; C—hydroxybenzoic acid; D—vanilic acid; E—syringic acid; spectra of standards were scaled to the droplets' once for better comparison.

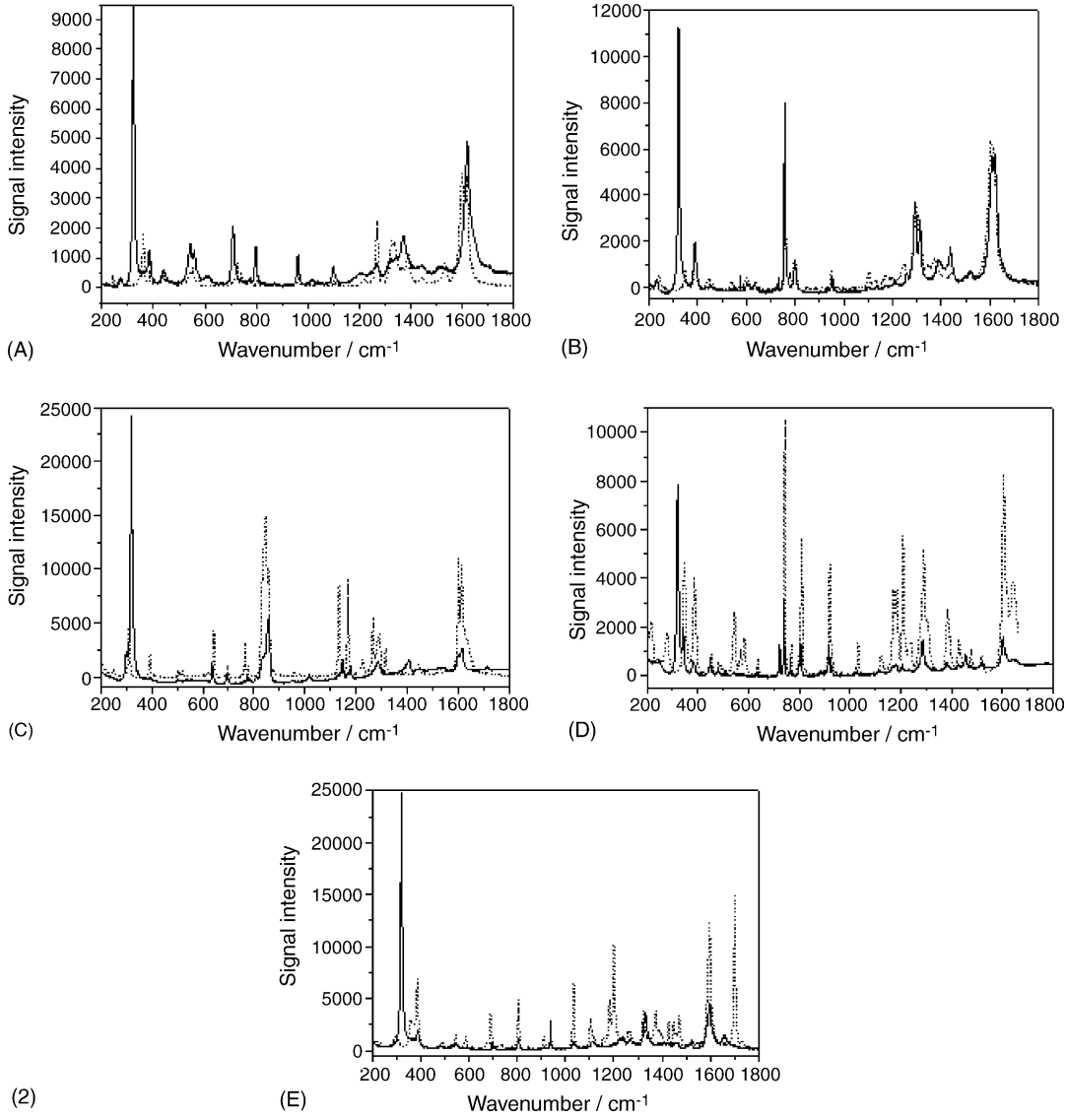

Fig. 5. (*Continued*).

4.2. HPLC measurements

To demonstrate the applicability of the flow-through microdispenser as an interface for μ-HPLC, FTIR and Raman measurement, a mixture of five acids (2000 ppm concentration each) was separated on RP-HPLC column with water-methanol eluent using set up and conditions described in the experimental section (Fig. 3). Measurements done for the 3,4-dihydroxybenzoic acid showed that in the chromatographic system used the analyte concentration in the peak maximum was approximately six times lower than in the sample.

Effluent splitting after the column was necessary since the flow rate used for the HPLC separation was too high for the proper dispensing procedure—with higher flow rates solvent was leaking from the nozzle hindering thus the droplets emission. The chromatographic trace consisted of solid deposits, each resulting from the dispensing of approximately 30 droplets of ca. 60 pl. The 3,4-dihydroxybenzoic acid's deposit is shown on Fig. 4. As can be seen, a high concentration leads to a heterogeneous deposition of the analyte, mostly concentrated on the edges of the deposit. However, homogeneous deposits could also be obtained for lower concentrations of the analyte.

During the dispensing procedure the ejected droplets were deposited on a CaF_2 substrate and evaporated at room temperature and atmospheric pressure, leaving behind a solidified trace of the chromatogram from which clear IR and Raman spectra of crystal spots of all five acids could be measured. These spectra, taken from the same deposits for both detection techniques, as well as the spectra of the standards of analyzed acids in their solid form are shown on Fig. 5. Comparison of both spectra clearly shows that despite the slight differences between spectra of standards and those obtained for analytes' deposits, all compounds could be unambigu-

ously identified. In the FTIR measurements (Fig. 5(1)), the spectral range is limited due to the use of a CaF_2 substrate, which only allows 100% transmission above 1025 cm^{-1}. In the comparison of the FTIR spectra of the analytes with those of the pure reference substances, slight shifts in the band positions can be observed, probably due to the effect of remaining water retained within the newly formed crystals as a result of the fast solvent evaporation. The Raman measurements (Fig. 5(2)), on the other hand, allow a wider spectral range since the substrate shows no limitations in this case; only the characteristic sharp peak at 322 cm^{-1} is observed in the spectra of the analyte deposits. The reference spectra were recorded by placing the pure substances in their crystalline form in a glass plate. When comparing both sets of spectra (analyte versus reference), again small shifts in the peak positions can be observed together with some more relevant differences, especially in the case of the syringic acid. It is possible that the crystallization conditions (fast solvent evaporation at room temperature) led to a different phase than that present in the reference crystal; the presence of another crystalline phase is easily detected by Raman spectroscopy.

In order to evaluate sensitivity of the method, it was compared with the typical flow-through cell IR set-up. Semi-quantitative measurements showed that the sensitivity of the described solvent-elimination IR detection for the investigated compounds was at least one order higher than for the flow-through IR detection cell. This finding is promising considering the fact that only a very small part of the solution compared to flow-cell measurements was used when measuring the deposits. Taking into account the split ratio and the amount of solution actually deposited it gets clear that only about 0.001% of the solution eluting from the column was deposited for measurements.

The dispensing conditions were stable during the whole separation time for both—isocratic and gradient modes, proving the ability of the dispenser to work properly under any separation conditions. Care however must be taken that in the gradient mode mixed solutions are well degassed or that the degasser unit is used in the system. Air bubbles can be formed as a result of mixing water with organic solvents. These air bubbles distort proper functioning of the dispenser and frequently prevent droplet ejection.

5. Conclusion

The application of a piezoactuated flow-through microdispenser as a solvent elimination interface for μ-HPLC-IR and Raman was shown. The presented interface enabled fully automated on-line deposition of HPLC effluents consisting of water–methanol mixtures. The chosen conditions allowed fast separation and deposition of the five phenolic acids of interest enabling their interrogation by IR and Raman spectroscopy. The proposed solvent elimination method is easy to handle, fast and does not require complicated instrumental set ups.

The continuous dispensing of the HPLC effluent takes place over the spectroscopic plate placed on the *x*, *y*-stage, which operation is controlled by computer and enables its exact positioning and movement under the dispenser. This ensures obtaining a precise droplet pattern, as sub-fractions of the column effluent are continuously transferred onto a target plate. Obtained deposit sample spots are then analysed by infrared and Raman microscopic techniques, which proved to have different advantages and drawbacks. Whilst FTIR provides more reproducible but less sensitive measurements, Raman spectroscopy allows for the detection of minute amounts of samples. Further improvement of the proposed concept may be achieved by reducing dispersion during chromatographic separation and by a general reduction of the column diameter and corresponding flow rates. Currently the flow rate required for the deposition procedure represents only 0.01% of the eluent flow passing through the dispenser. It may be expected that significant improvements in terms of concentration sensitivity can be achieved when using capillary-LC techniques instead of a μ-LC and flow splitting. Capillary-LC technique, which is characterised by lower flow rates, better efficiency and lower system's dead volume than μ-HPLC system would enable to make use of a higher portion of the eluting sample. A combination of both FTIR and Raman techniques in a single microscope has recently been shown which furthermore facilitates the application of the method on a routine basis.

Acknowledgements

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