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Investigation of mandelic acid bonding on Pirkle type chromatographic stationary phases by Raman spectroscopy

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Abstract

The bonding of mandelic acid enantiomers has been studied on benzene–leucine, dinitrobenzene–leucine and dinitrobenzene–phenylalanine type chiral stationary phases connected to zeolite A supports. The π-donor, π-acceptor and H-bonding interactions responsible for diastereomer pair formations can be studied under quasi in situ chromatographic conditions by Fourier transform Raman and surface enhanced Raman spectroscopic techniques. Structural differences between diastereomer pairs result in observable spectral differences at a phase load of approx. 50%. It was shown that the decreasing π-acceptor character of the phase is associated with its increasing capability of H-bond formation. Correlating spectral data to chromatographic results it can be concluded that, in addition to H-bonding as well as to π-donor–π-acceptor interactions, steric hindrances due to bulky moieties of either the stationary phase or the analyte molecules are of importance in successful separations.

Keywords: Chiral stationary phases, LC; Raman spectroscopy; Enantiomer bonding; Mandelic acid

1. Introduction

The application of *N*-(3,5)-dinitrobenzoyl amino acid-based chiral chromatographic stationary phases (CSPs) resulted in a real breakthrough in the separation of low-molecular-mass α-hydroxy acid and amino acid enantiomers [1–3]. Such types of stationary phases can form charge transfer (CT) – or π-donor–π-acceptor – complexes with the analyte molecules.

Early chromatographic works already paid attention to the formation of CT complexes during separation [4–6]. Later works also emphasized the importance of π-donor–π-acceptor interactions. At the same time, the role of H-bonding as well as the presence of steric repulsive forces were found to be important as well [7–10]. The significance of the structure/order of the support material was also recognised, since the order of support surface can increase the order of the chromatographic stationary

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phase as well. In this way, the formation of clusters responsible for non-enantioselective separations can be avoided [11].

In order to understand the nature of chiral separations, the structure of the stationary phase–analyte complex, the connection of functional groups as well as the types of parameters influencing retention have to be known. Chromatographic investigations aimed at the understanding of these problems face with the difficulty that processes taking place in the chromatographic system as a ''black box'' can only be investigated indirectly from chromatographic measurement data. More recently, developments of surface analysis techniques – especially Raman spectroscopy and Raman microscopy – opened new horizons in the study of chromatographic systems as well. Since the Raman scattering of the support is negligible, the advantages of Raman spectroscopy in the study of CSP–analyte interactions can be exploited.

The authors used Raman spectroscopy for the first time to investigate stationary phase–analyte interactions under quasi in situ chromatographic conditions [12]. In the present work the role of steric hindrances is discussed and it is shown that a correlation exists between spectroscopic and chromatographic data. The chromatographic system used showed a high selectivity towards the separation of enantiomer pairs. In addition, the possibility to record Raman spectra after the main steps of CSP synthesis was a further advantage over the use of commercial stationary phases.

2. Experimental

2.1. Chromatographic investigations

Separation of mandelic acid enantiomers from an aqueous solution of the racemic mixture of 1 mg cm^{-3} concentration was carried out on benzene–L-leucine (LeuB), *N*-(3,5)-dinitrobenzene–L-leucine (LeuDNB) and *N*-(3,5)-dinitrobenzene–L-phenylalanine (PhDNB) type stationary phases connected to a zeolite A support. The schematic structures of the phases used are shown in Fig. 1. Packings of 30 mm×4 mm have been prepared using 0.38 to 0.40 g stationary phase (BST, Budapest, Hungary). Optimal

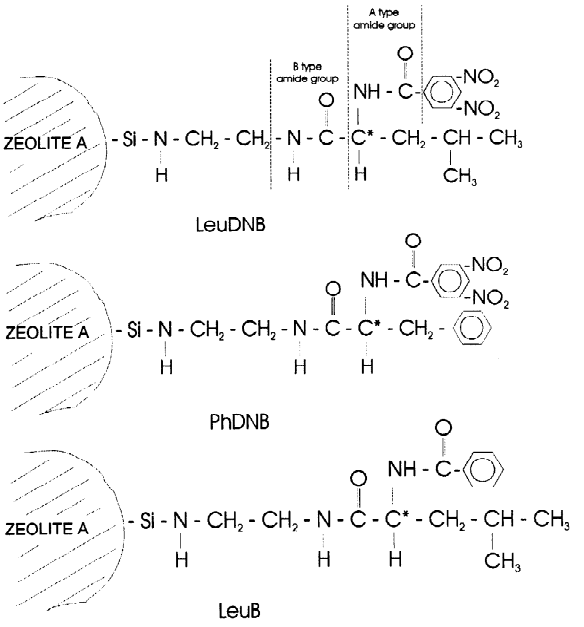

Fig. 1. Schematic structures of the stationary phases studied.

separations were achieved with a 20 m*M* phosphoric acid solution containing 1 m*M* *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (cetrimide) as pair forming reagent (Merck, Germany). Eluent pH was adjusted to 4.5 with triethylamine (Sigma–Aldrich, Hungary), and a flow-rate of 0.5 cm^3 min^{-1} was used. Water as solvent was prepared by distillation of a 1 m*M* $KMnO_4$ solution. Separations were carried out by means of a Merck–Hitachi modular system high-performance liquid chromatography (HPLC) apparatus consisting of an L-6200 type gradient pump, a 20 μl volume Rheodyne Model 7125 type injector, and an L-4500 type diode array detector. Data collection and processing was performed by a Model D-6500 type software package.

2.2. Sample preparation for Fourier transform Raman spectroscopic studies

Since under realistic chromatographic conditions (with 0.1 m*M* analyte/100 g stationary phase) no detectable surface concentration could be achieved, some 50% of the available active sites (30 m*M* analyte/100 g stationary phase) were covered using 5 mg cm^{-3} concentration aqueous analyte solutions.

Although in chromatographic terms it is an overloaded system, it does not influence the study of CSP–enantiomer interactions. Fourier transform (FT) Raman spectra of the air-dry samples were recorded by means of a Bio-Rad (USA) FT-Raman spectrometer using Nd–YAG laser at 500 mW power. Three thousand spectra were co-added at a resolution of 2 cm^{-1} using 180° sample geometry. The recorded spectra were corrected to the white light background.

2.3. Sample preparation for surface enhanced FT-Raman spectrometric and Raman microscopic measurements

For surface enhanced FT-Raman studies 10-mg portions of the samples (with 1–2 μm CSP particle size) were mixed with 3×5 μl silver sol prepared after Lee and Meisel [13]. Before use, the sol was concentrated 10 times by centrifugation (at 4000 rpm for 30 min). The spectra were recorded 15 min after sol addition. Raman microscopic investigations were carried out on samples prepared in a similar way. Very small amounts of samples were placed on a polished metal surface of an Olympus BHSM microscope, equipped with 10×, 20× and 50× objectives. The microscope is part of a Renishaw 1000 type Raman microscope system, which also includes a monochromator, a filter system and a charge-coupled device (CCD). Excitation was carried out by a Spectra-Physics Model 127 He–Ne (633 nm) and a diode (785 nm) laser. To increase the signal-to-noise ratio, 24 spectra were accumulated at a resolution of 2 cm^{-1}. Evaluation of spectra were carried out after a Savitzky–Golay noise filtration followed by a noise-minimisation step using the modified NIPALS (non-linear iterative least-squares) [14,15] program.

3. Results and discussion

The FT-Raman spectra of the CSP–enantiomer complexes studied show significant differences in the νNH and νCH stretching bands as well as in the 1010 to 995 cm^{-1} range of analyte ring vibrations. The νNH band of the 3375–3275 cm^{-1} range belonging to CSP amide groups can be resolved to at least four Lorentz type components using the Jandel Scientific Peakfit 2.0 program. The band positions of the resolved νNH Lorentz bands as well as the base width of the recorded νNH band are given in Table 1. It can be seen that Lorentz peak maxima shift to higher frequencies as a result of enantiomer bonding to the CSP. At the same time, the base width of the νNH band slightly increased. According to literature data [16,17], the νNH band of the amide group shows a downshift and a band broadening upon H-bonding, while the increase of the *trans*-isomer

Table 1
Band base widths and Lorentz peak positions of the νNH bands for various CSP–analyte complexes

CSP alone		D-Mandelic acid+CSP		L-Mandelic acid+CSP		Type of CSP
ν (cm^{-1})	Base width	ν (cm^{-1})	Base width	ν (cm^{-1})	Base width	
3315	68	3321	70	3319	77	LeuDNB
3322		3328		3324		
3328		3336		3330		
3337		3344		3337		
3315	69	3321	71	3314	76	PhNB
3323		3328		3326		
3329		3335		3333		
3337		3343		3340		
3305	68	3313	73	3317	82	LeuB
3321		3320		3324		
3326		3326		3328		
3334		3330		3334		
3344		3340		3344		

character results in a frequency upshift. The spectral pattern of the νNH band is, therefore, the result of these two effects. It can also be seen from Table 1 that the νNH band shows an upshift and a little broadening with the decreasing π-acceptor character of the CSP. Thus, it can be concluded that the ability of H-bond formation is increased with the decreasing π-acceptor character of the phase, and – at the same time – *trans*-isomerism becomes stronger in the amide groups. It should be noted that the occurrence of H-bonding interactions can also be supported by the broadening of the analyte νCOO and the CSP amide I bands associated with a slight shift in peak positions to lower frequencies. Since these spectral regions give no information on the strength of bonding to the CSP, interpretation of the relevant spectral details are omitted. Figs. 2 and 3 indicate spectral differences (in band widths and band positions) between the CSP–enantiomer complexes in the 3200–3100 cm^{-1} and the 1010–995 cm^{-1} ranges. The biggest difference can be observed for the PhDNB stationary phase, while the smallest one for the LeuB phase.

In direct separations enantioselectivity is ensured by the stability difference between the CSP–analyte complexes. A complex is more stable if more and/or stronger interactions are formed between the functional groups due to symmetry and/or steric reasons. In the case of Pirkle type stationary phases the amide groups can form dipole–dipole as well as H-bonding interactions with the analyte molecules. π-Donor and π-acceptor interactions between the stationary phase and the analyte molecules are thought to be the most important during separation [18]. In fact, no separation could be possible without these forces. Greater differences in the stability of the CSP–enantiomer complexes should result in greater differences in the spectral patterns as well due to differences in symmetry. If spectral differences can really be correlated with stability differences of the CSP–analyte complexes, then the best separation can be anticipated with the PhDNB stationary phase. Experimental data such as separation factor (α), asymmetry factor (A_s) and theoretical plate number (N) calculated from the chromatogram are indeed in harmony with the conclusions drawn from spectroscopic patterns (Table 2). Although on LeuB stationary phase a higher plate number was obtained for D-mandelic

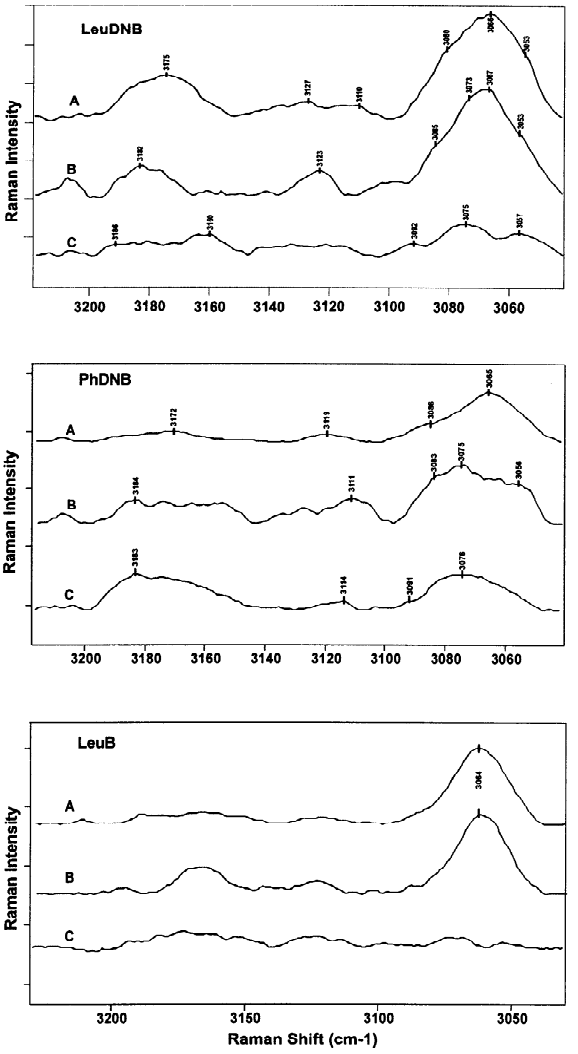

Fig. 2. FT-Raman spectra of the CSP–analyte complexes in the 3250–3000 cm^{-1} range. (A) D-Mandelic acid+CSP, (B) L-mandelic acid+CSP, (C) CSP alone.

acid than on the PhDNB phase, the values of A_s and N for L-mandelic acid cannot be calculated due to the split of the chromatographic peak. At the same time, however, unfavourable A_s and N values were obtained with the LeuDNB phase, where the π-donor–π-acceptor interactions (which are thought to be the predominant ones for separation) are the strongest. Since all H-bonding interactions are of similar type as a result of the uniformity in nature of the functional groups, the steric hindrance due to the

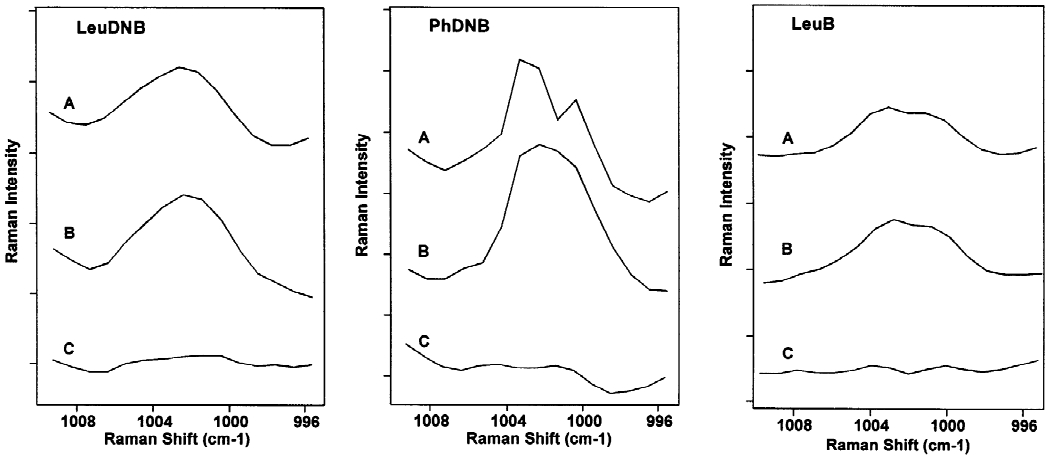

Fig. 3. FT-Raman spectra of the CSP–analyte complexes in the 1010–995 cm^{-1} range. (A) D-Mandelic acid+CSP, (B) L-mandelic acid+CSP, (C) CSP alone.

presence of the benzene ring on the stationary phase can play a significant role in the separation of the analyte antipodes.

With the data of Table 2 it should be noted that the CSPs used were developed for industrial purposes. The fact that the unfavourable mechanical properties do not allow a high pressure drop on the column (the particle size is in the 2–10 μm range instead of the desired 1–2 μm one) obviously reduces column efficiency. The use of the lipophylic buffer and the presence of the ion-pair forming agent results in further band broadening. Although the investigations aimed primarily at the influence of support crystallinity on separation, retention data can advantageously be used for the interpretation of Raman spectra. The preparation procedure as well as the applicability of the stationary phases for the separation of low-molecular-mass α-hydroxy and amino acids has already been published elsewhere [19,20].

Table 2
Separation factor (α), asymmetry factor (A_s) and theoretical plate number (N) values for L(1)- and D(2)-mandelic acid calculated from retention data

Type of CSP	α	N_1	N_2	A_{s1}	A_{s2}
LeuDNB	2.67	31	56	3.34[a]	2.23
PhDNB	5.38	58	262	1.46	1.87
LeuB	3.65	[b]	491	[b]	1.67

[a] Calculated from the highest intensity band.
[b] Cannot be calculated.

To demonstrate the role of steric hindrance during separation, the Surface Enhanced Raman (SERS) spectra obtained by a diode laser (785 nm) and a Nd–YAG laser (1064 nm) are given in Figs. 4 and 5, respectively. Although the 1616 ± 10 cm^{-1} range (which is characteristic of H-bonding interactions as well) does not provide much information, the SERS spectra of the CSP–analyte complexes show band splitting, band broadening and shift of the peak frequencies as compared to the spectrum of the pure CSP. The 1100–970 cm^{-1} range of the aromatic ring vibration, however, is highly informative. The relative intensities of the 1029 ± 2 cm^{-1} band (CSP) and the one at 1001 ± 3 cm^{-1} (analyte ring vibration) show considerable changes. In order to compare band intensities, the bands of the SERS spectra in the 1450–800 cm^{-1} range were ratioed to the 1150 (with 785 nm laser) and the 1448 cm^{-1} (with 1064 nm laser) bands selected as standards. The relative intensity ratios for all CSP–analyte complexes investigated are summarised in Table 3. The data for the PhDNB phase show extreme values.

In order to interpret the changes in band ratios, the connection of silver to the CSP–analyte complex should be considered first. It is commonly accepted that the SERS spectrum is the consequence of

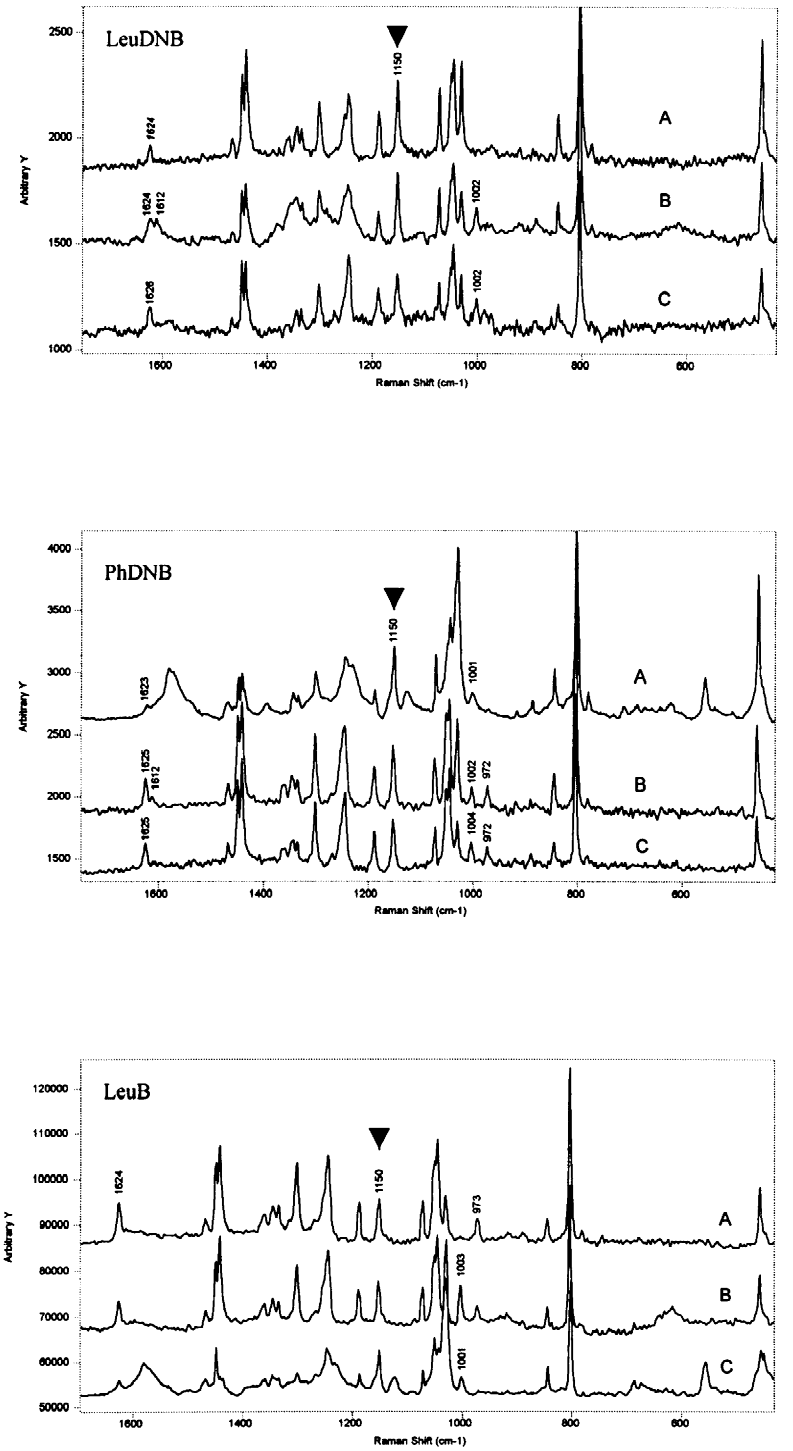

Fig. 4. Surface enhanced Raman spectra of the CSP–analyte complexes (with 785 nm diode laser). (A) CSP alone, (B) L-mandelic acid+CSP, (C) D-mandelic acid+CSP.

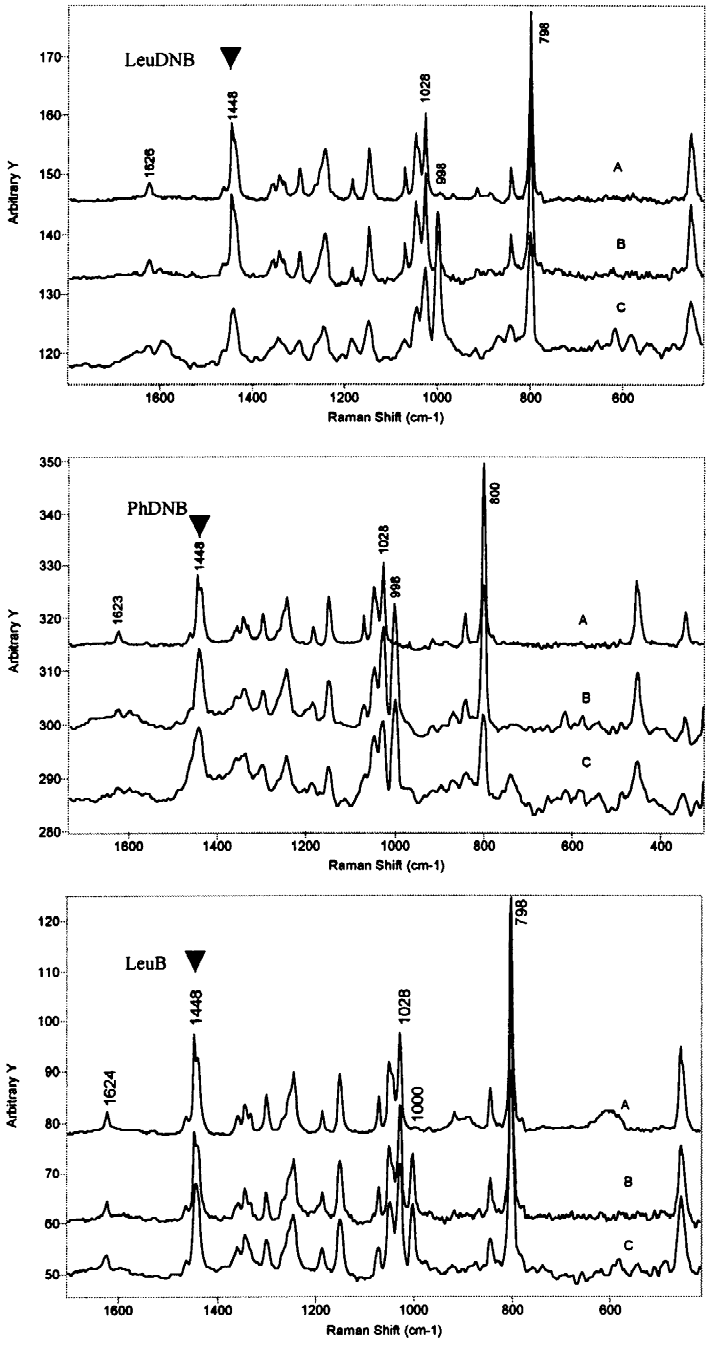

Fig. 5. Surface enhanced Raman spectra of the CSP–analyte complexes (with 1064 nm Nd–YAG laser). (A) CSP alone, (B) L-mandelic acid+CSP, (C) D-mandelic acid+CSP.

Table 3
Relative band intensities calculated from SERS spectra

Type of CSP	Diode laser (785 nm)								
	I_{1030}/I_{1150}			I_{1003}/I_{1150}			I_{802}/I_{1150}		
	CSP	CSP+L-mandelic acid	CSP+D-mandelic acid	CSP	CSP+L-mandelic acid	CSP+D-mandelic acid	CSP	CSP+L-mandelic acid	CSP+D-mandelic acid
LeuDNB	1.25	0.64	1.00		0.43	0.60	2.27	3.61	3.45
PhDNB	1.35	1.39	0.82	0.26	0.31	0.41	3.70	3.46	3.12
LeuB	2.00	1.09	1.39		0.82	0.44	2.90	3.75	3.55
	Nd–YAG laser (1064 nm)								
	I_{1028}/I_{1448}			I_{998}/I_{1448}			I_{800}/I_{1448}		
	CSP	CSP+L-mandelic acid	CSP+D-mandelic acid	CSP	CSP+L-mandelic acid	CSP+D-mandelic acid	CSP	CSP+L-mandelic acid	CSP+D-mandelic acid
LeuDNB	0.95	1.35	1.45		0.62	1.75	2.00	2.38	2.22
PhDNB	1.11	1.33	1.11		1.64	1.37	2.50	1.89	1.27
LeuB	0.98	1.30	1.75		0.76	1.09	2.27	2.63	3.12

chemisorption between the silver surface and the nucleophilic moiety of the molecule, thus the spectral information is characteristic of the chemisorbed layer. Bands due to Ag–N and Ag–O bonds appear between 250 and 210 cm^{-1} in the SERS spectra [21]. Strong bands were observed at 240 and 214 cm^{-1} with both lasers which can be assigned to Ag–N and Ag–O stretching vibrations, respectively. In terms of band shifts the SERS spectrum shows no differences as compared to the FT-Raman spectrum. Differences can be obtained in band intensities and band ratios, only. It should be noted that analyte bands can only be found in the SERS spectrum when low-energy lasers (Nd–YAG and diode lasers) are used. This phenomenon is explained by the photo-induced desorption of the analyte when lasers of higher energy are used [17]. In our judgement no desorption occurred in the systems studied, because an increase in analyte band intensities was observed when the spectra were recorded with the use of the Nd–YAG laser on samples previously examined by the He–Ne laser. On the basis of these, it can be concluded that high-energy lasers give information mainly on the stationary phase, while with lower laser energy most of the information is associated with the CSP–analyte complex (i.e., with the surface). Since mandelic acid gives surface enhancement neither on silica layer [5 μg analyte per thin-layer chromatography (TLC) spot] nor in solution phase, it can be said that silver is connected to the surface through the $-CH_2$-NH-C(=O)-C*≡ group (B type amide, see Fig. 1). This is because the π-donor–π-acceptor interaction between the analyte and the stationary phase can result in H-bonding with the ≡C*-NH-C(=O)-DNB (A type amide, see Fig. 1) group, only. On the other hand, the π-donor–π-acceptor interaction will increase the acidity of mandelic acid and, via this, the interactions with A type amide are strengthened. It means that the connection between the A type amide and the analyte is not disrupted when silver is applied to the surface. The local electric field around silver connected to B type amide, however, affects the analyte in spite of the fact that no direct connection exists between them. Aroka and Buljaski [21] found a broadening of the amide I band and a significant shift in band position during the SERS detection of thymine on silver electrodes and on silver island films. Band shifts were also observed in the range of ring vibrations, too. On the other hand, Klug [22] found no considerable band shifts in the SERS spectra of phthalates recorded on oxidised aluminium surfaces. A similar situation was experienced with the SERS detection of *p*-dimethylamino-benzylidene rhodanine

on a Kieselgel 60 TLC layer [23]. Thus, connection to silver does not necessarily mean drastic changes in the SERS spectrum as compared to the situation with the normal Raman spectrum.

In accordance with the surface selection rule, vibrations having a component perpendicular to the silver surface are Raman active, only. In our earlier study structure estimation based on the calculation of conformational energy minima was made using the Desktop Molecular Modeler version 2.0 program [12]. On the basis of this, it can be concluded that vibrations perpendicular to the silver surface are perpendicular to the support surface, as well. From the point of view of separation, the most important is the position of the *N*-(3,5)-dinitrobenzoyl moiety and the A type amide group. The highest band intensity ratio in the ranges of ring vibrations (at 1030 and 1028 cm^{-1}) as well as the ring out-of-plane vibrations were obtained for the PhDNB stationary phase (see Table 3). Due to the presence and steric effect of the bulky phenyl group, the dinitrobenzoyl group is almost perpendicular to the surface, providing an easy access of the analyte to the DNB group. The intensity ratios of the ring out-of-plane vibrations (which are especially sensitive to π-donor–π-acceptor interactions) show significant changes as referenced to the CSP when the analyte is bonded to the surface. The band ratio decreased for the PhDNB phase, while an increase can be observed for the LeuDNB and LeuB stationary phases. Among the possible reasons are the conformational change as well as the change in polarisation of the interacting groups. The polarisability of the strongly interacting benzoyl groups was reduced. A decrease in the band ratio can also be observed in the SERS spectrum when the angle of the interacting groups to the surface is decreased. Therefore, it is more reliable to consider the differences in band ratios of the two CSP–enantiomer transitional complexes. The differences in band ratios of the out-of-plane vibrations show a close correlation with the separation coefficient. Thus, it can be concluded that the differences in band ratios are proportional to the differences in stability of the transitional complexes which is in harmony with chromatographic data. The biggest difference in band ratios (0.34 with the diode laser and 0.62 with Nd–YAG laser) and the highest separation factor (5.38) was obtained for the PhDNB phase.

4. Conclusion

Through the example of mandelic acid bonding on Pirkle type stationary phases it was demonstrated that the bonding mechanism can be studied with low energy excitation lasers and by the surface enhanced Raman spectrometric technique. The ability of H-bond formation of the stationary phase is increased with the decrease of its π-acceptor character. In addition to forces holding together the transitional complex, steric hindrances are of importance in successful separations. In the SERS spectra of the transitional complexes the relative band intensities of the ring out-of-plane vibrations show correlation with retention data.

This work is part of the efforts aimed at the elaboration of a Raman spectrometric method capable of predicting information necessary to select/design suitable stationary phases.

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