SELECTED APPLICATIONS OF RAMAN AND IR SPECTROSCOPY IN CHROMATOGRAPHY

Summary of Ph.D. THESIS

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1. Introduction

In practice of analytical applications of chromatography there is a wide choice of detection methods, whereas none of these methods ensure direct and reliable identification of the separated substances. There are numerous examples proving that „identification" based on retention parameters can be misleading, since the chromatographic parameters applied during separation can result in peaks eluted together (with identical retention times) if two (or more) substances have nearly the same absorption-desorption properties under the given measurement conditions. Thus, one of the application areas of detection methods that are capable of structural identification can be examination of peak purity.

Understandably, „identification" based on retention parameters is possible only if the corresponding reference materials are available. However in case of samples of unknown composition or samples containing unknown compounds as well, one needs again a detection method or methods capable of determining the molecular structure.

Although direct identification can be performed on collected fractions of the separated components one-by-one, examining then by one or more methods of structural elucidation, it has become the demand of our time to apply coupled techniques offering „on-line" and „real-time" measurement. The term „on-line" means such an integrated unit of chromatographic and spectroscopic instruments that can ensure the real-time feature of measurement, i.e. detection allowing structural identification simultaneously with separation of components.

The coupled (or hyphenated) techniques have the advantages that there is no need to collect fractions and do repeated chromatographic measurements with reference compounds (saving time); the possibility of contamination during collection and storage of fractions is averted and, furthermore, it is possible to gain reliable qualitative information for sample identification.

For the purpose of structural identification those measurement methods should be used in the first place which are suitable for finger-printing of the substance at the molecular level, i.e. offer molecule-specific identification. Such a possibility is offered (beside mass spectrometry and NMR spectroscopy) by vibrational spectroscopy as well, which is the specialty of the research laboratory where my work has been done. Thus the work to be presented here deals with applications of infrared (IR) and Raman spectroscopy combined with separation techniques. After a short overview of the possible coupling combinations based on literature sources, this dissertation presents, on the one hand, an application of conventional and surface-enhanced Raman spectroscopy to spots on chromatographic thin layers, and on the other, the problems arising in practice of HPLC-IR coupled technique, their possible ways of solution, and a specific implementation and application of the technique itself. We are also indicating some areas of chromatographic applications in which the HPLC-IR equipment assembled in our laboratory could be used with success.

2. Aims

Our intention has been to do research in two distinct areas of coupling of vibrational spectroscopy with chromatographic methods, namely: in the area of combining thin layer chromatography (TLC) with Raman spectroscopy, and in the area of HPLC-FTIR technique utilizing the flow-through cell method.

For TLC-Raman and TLC-SERS measurements we have chosen L-α-amino acids as the target compounds. Separation and identification of amino acids is a frequent task in biochemistry, and taking into account that these substances are not volatile, thin layer chromatography as a fast and simple separation method may be a convenient choice. At the same time, since these compounds are not UV-active (without derivatization), instead of UV detection some suitable vibrational spectroscopic (or other) method should be used. Furthermore, since from literature

data it is known that direct IR spectroscopic (DRIFTS or photoacoustic) measurement of sample components on TCL plates suffers from several problems (so that these measurements should not be made on the TLC plate), it seemed timely to investigate the possibility of Raman spectroscopic detection directly on the plates. Even more so, since there was some controversy in the literature regarding the right choice of wavelength for laser excitation in TLC-Raman measurements. We intended to examine the applicability of different TLC plates as well. On the one hand, when selecting the TLC plates, we were looking for layers that would add little background scattering to the spectra of the amino acid samples; on the other hand, one of the TLC plates selected for testing was specifically recommended (by its manufacturer) for TLC-Raman measurements. These thin layers were subjected to FT-Raman measurements with near-IR excitation and to Raman microspectroscopic measurements with excitation at three different visible wavelengths. Our aim with this and with the subsequent measurements on TLC spots of amino acids was to establish the best choice of exciting laser line and most convenient combination of TLC plate and excitation wavelength (regarding the smallest background scattering and lack of fluorescence). Among our aims was also to explore the circumstances that would allow surface enhanced Raman spectroscopic (SERS) measurements to be made on TLC spots, and examine the dependence of surface enhancement (i.e. the increase in scattering intensity of Raman bands) on excitation wavelength.

Although the HPLC-FTIR technique using the flow-through cell method or the eluent elimination method was first demonstrated in the same decade (the 1970s), since that time greater attention has been paid to the development of the latter. Concerning the flow-through cell method, the problems connected with the strong absorption bands of the moving phase (compared to the absorption of the separated components) have not been practically dealt with. During the past decades, however, the FTIR measurement technique has undergone significant development,

so our aim was to evaluate the present day possibilities in this respect by means of the up-to-date, high-speed, and high sensitivity FTIR equipment available in our laboratory. Besides, the articles published so far (dealing with applications of normal phase (NP) HPLC-FTIR measurements utilizing a flow-through cell interface) have presented only attempts employing IR-compatible solvents (mobile phases). Because of this another goal of ours was to perform NP-HPLC-FTIR measurements using conventional chromatographic mobile phase mixtures generally used during HPLC separations. Furthermore, although there are numerous review papers discussing the literature of both basic approaches of HPLC-IR measurements, none of them cites any paper that would clearly reveal the detection and identification limits attainable by the flow-through cell (FTC) method applied with the use of an *analytical* chromatographic column. Thus, we wanted to establish the attainable detection and identification limits during the separation of a mixture, a practically important chemical product, while using typical chromatographic mobile phase mixtures.

With the method of measurement specified above, we practically accept the problems stemming from the strong absorption of the mobile phase. In another section of our work we tried to sketch and find potential solutions to this problem whose detailed development could really solve this problem. Of these, the chemometric method that uses the series of spectra originating from HPLC-FTIR measurements as starting data, and yields (among others) the spectra of single separated components (free from eluent interference) as a result, in still under development.

3. Methods applied

The TLC plates and the amino acids adsorbed on TLC plates have been examined by means of Fourier transform Raman (FT-Raman) and microscopic dispersive Raman spectroscopy utilizing various near-IR and visible lasers for excitation. The TLC spots of a few amino acids have been examined by the method of surface enhanced Raman spectroscopy (SERS) as well, also with different laser excitations. The silver sol used in SERS experiments to generate surface enhancement was examined by scanning electron microscopy (SEM). Our existing digital spectral library was expanded with the Raman spectra recorded, and the spectra obtained during the TLC-Raman measurements were evaluated on the basis of spectral search using the extended data library.

The method of Fourier transform infrared (FTIR) spectroscopy has been used for on-line detection of chemical components separated by normal phase (NP) high performance liquid chromatography (HPLC). On-line detection has been achieved by means of a coupled HPLC-FTIR instrument interfaced by a flow-through cell. The time dependence of the eluted quantities of the various components has been monitored by Chemigram-type IR chromatograms and Gram-Schmidt reconstructions.

The series of IR spectra belonging to simulated and real on-line HPLC-FTIR measurements were analyzed by chemometric methods (PARAFAC, PARAFAC2) based on factor analysis, in order to extract the individual spectra of the separated components (the spectral profile) as well as the time profile and concentration profile.

4. New scientific results

Among many possible applications of Raman and IR spectroscopy coupled with separation techniques, we have made examinations connected to TLC-Raman, TLC-SERS and HPLC-IR applications, and have achieved the following results.

I/A. TLC-Raman investigations:

I/A/1. Based on TLC-Raman measurements of seven essential amino acids (Gly, Ala, Ser, Val, Pro, HO-Pro, Phe), it has been established that excitation of the spectra with the 1064 nm Nd:YAG laser has definite background and Signal/Noise advantages over the 532, 633 and 785 nm excitations of a Raman microscope.

I/A/2. It has been shown that Raman detection and identification of weekly scattering aliphatic amino acids by FT-Raman spectroscopy (with the 1064 nm excitation) and computer assisted spectral search require sample amounts in the 100 μg range.

I/A/3. It has been pointed out that the *Kieselgel 60* type plate has the smallest background scattering among the examined TLC plates, and the Raman spectra of amino acids adsorbed on it are very similar to those of their aqueous solutions, where the concentration of solution is equal to the solubility of the amino acid.

I/B. TLC-SERS investigations:

I/B/1. It has been established that surface enhanced Raman spectra (*i.e.* SERS spectra) of TLC spots of amino acids can be generated by adding Ag-sol on top of the analyte spot and keeping the sample wet during the measurement.

I/B/2. It has been established that the magnitude of attainable enhancement is 10-1000 fold, greatest with the 532 nm exciting laser and smallest with the 1064 nm exciting laser.

I/B/3. Comparing the results of the TLC-Raman and TLC-SERS measurements, we have pointed out that the TLC-SERS spectra are less suitable for analyte identification (especially at high lateral resolution of a Raman microscope) than those conventional Raman spectra obtainable with a (NIR) FT-Raman instrument equipped with 1064 nm exciting laser.

II. Chemometric analysis of HPLC-IR data

In order to facilitate the interpretation of the results of HPLC-IR measurements done without eluent elimination, we have examined the applicability of certain chemometric methods.

II/1. In the course of analyzing *simulated* HPLC-IR data by chemometric methods, PARAFAC and PARAFAC2, it has been pointed out that PARAFAC2 can calculate spectra of the chemical compounds (that free from eluent) properly; the match index between the calculated and reference spectra is 95-98 %. The calculated time profile and concentration profile were recovered appropriately, and their proper linear combinations have also shown satisfactory agreement with the simulated IR chromatograms.

II/2. In case of measured HPLC-IR data containing molecular interactions and measurement noise, the algorithm of PARAFAC2 method had to be improved, and the "Objective Subtraction of Solvent Spectrum with Iterative Use of PARAFAC2" (OSSS-IU-PARAFAC2) method was developed.

II/3. Analysing measured HPLC-IR data by the OSSS-IU-PARAFAC2 method, it has been pointed out that the method separates spectra of chemical compounds properly, but elimination of the eluent is not complete. The linear combinations of the calculated time profile and concentration profile has shown satisfactory agreement with the measured IR chromatograms.

III. HPLC-IR investigation of β-cypermethrin isomers

III/1. It has been demonstrated that on-line coupling of *analytical* HPLC with a research grade FTIR spectrometer by means of a flow-through cell is a viable approach to major component detection and identification of organic chemical products.

III/2. In contrast to the related main-stream literature, it has been shown that the usual chromatographic mobile phases containing polar modifiers can also be used during flowcell-interfaced HPLC-IR measurements (under certain conditions).

III/3. It has been shown that chemigram-type IR chromatograms are more suitable for major component detection in HPLC-IR measurements than Gram-Schmidt reconstructed chromatograms (which is a highly successful method in GC-IR measurements).

III/4. It has been established that quantitative determination of the two major diastereomers in the β-cypermethrin sample using the chemigram type IR chromatogram shows good linearity in the concentration range from 0.3-4.0 mg/ml.

III/5. It has been established that the HPLC-IR detection limit for the β-cypermethrins examined is 0.3 mg/ml when dichloromethane/n-hexane, tetrahydrofuran/n-hexane or isopropanol/n-hexane mobil phase is applied, and it is estimated to be 0.1 mg/ml in case of acetonitrile/n-hexane mobil phase.

III/6. It has been established that HPLC-IR identification limit still allowing spectral discrimination of β-cypermethrin diastereomers is at an injected concentration of 1 mg/ml.

5. Publications

5.1. Publications based on the Ph.D. work

Papers in journals and proceedings volumes:

1. **K. István**, G. Keresztury:
   TLC-Raman and TLC-SERS measurements of compounds containing amino and carboxyl groups
   Proceedings of XVIIIth International Conference on Raman spectroscopy, p.313, 2002

2. **K. István**, G. Keresztury, A. Szép:
   Normal Raman and surface enhanced Raman spectroscopic experiments with thin layer chromatography spots of essential amino acids using different laser excitation sources
   Spectrochim. Acta., **59A,** 1709-1723, 2003 IF: 1.315

3. **K. István**, G. Keresztury, J. Fekete:
   A normal phase HPLC/FT-IR detection and identification of β-cypermethrins by the Flow-Through Cell method: advantages and limitations
   J. Liq. Chromatogr. R.T., XXX, YYY-YYY, 2004 (accepted) IF: 0.709

4. **K. István,** R. Rajkó, G. Keresztury:
   Towards the solution of the eluent elimination problem in HPLC-IR measurements by chemometric methods
   Anal. Chim. Acta, 2004 (submitted)

Oral and poster presentations:

1. **István K.**, Keresztury G.:
   Aminosavak detektálása kromatográfiás vékonyrétegeken FT-Raman spektroszkópiai módszerekkel
   MTA IV. Doktori Kémiai Iskola, Mátraháza, 2001. május 20-22.

2. **K. István**, G. Keresztury:
   Detecting amino acids on TLC plates by FT-Raman spectroscopy?
   1st International Conference on Advanced Vibrational Spectroscopy, Turku, Finland, 19-24 Aug., 2001; Poster No.: P14.172

3. **K. István**, G. Keresztury:
   TLC-Raman and TLC-SERS measurements of compounds
   containing amino and carboxyl groups
   VIIIth International Conference on Raman Spectroscopy,
   Budapest, Hungary, 25-30 Aug., 2002; Poster No.: 70/M

4. **István K.**, Rajkó R., Keresztury G.:
   A HPLC/FT-IR csatolt rendszer működési elvei,
   problémák és a potenciális megoldás(ok)
   46. Magyar Spektrokémiai Vándorgyűlés, Szeged, 2003. jún. 30 – júl. 2.

5. **K. István**, R. Rajkó, G. Keresztury:
   Working principles of an HPLC/IR system: problems and a possible solution to obtaining useful analytical information
   Advances in Chromatograhpy and Electrophoresis-Conferentia Chemometrica, Budapest, Hungary, 27-29 Oct., 2003, Poster No.: P13

6. R. Rajkó, **K. István**, G. Keresztury:
   Towards the solution of the solvent/eluent problem in HPLC/IR (FTC) determination by chemometric methods
   Advances in Chromatograhpy and Electrophoresis-Conferentia Chemometrica, Budapest, Hungary, 27-29 Oct., 2003, Poster No.: P57

7. R. Rajkó, **K. István**, G. Keresztury:
   Towards the solution of the solvent/eluent problem in HPLC/IR (FTC) determination by chemometric methods
   VI. Nemzetközi Élelmiszertudományi Konferencia,
   Szeged, 2004. máj.21-22., No.: 22

8. Rajkó R., **István K.**, Keresztury G.:
   HPLC/FT-IR mérések és kemometriai szemléletű megoldásuk
   47. Magyar Spektrokémiai Vándorgyűlés (Vegyészkonferencia 2004), Balatonföldvár, 2004. jún. 30-júl. 2.

9. R. Rajkó, **K. István**, G. Keresztury:
   Another look at the self-modeling curve resolution (SMCR) of spectral data
   9th Chemometrics in Analytical Chemistry, Lisbon, Portugal, 20-23 Sept., 2004, Poster Session 3, [Abstract 153].

5.2. Related publications

1. R. Rajkó, **K. István**, G. Keresztury:
   Analytical solution for determining feasible regions of Self-Modeling Curve Resolution (SMCR) method based on computational geometry
   J. Chemometr., 2004 (submitted)

5.3. Other publications

Papers in journals and proceedings volumes:

1. C.Y. Panicker, H.T. Varghese, A. John, D. Philip, **K. Istvan**, G. Keresztury:
   FT-IR, FT-Raman and SERS spectra of 4-aminosalicylic acid sodium salt dihydrate
   Spectrochim. Acta, **58A,** 281-287, 2002 IF: 1.315

2. **K. István**, G. Keresztury, O. Berkesi, T. Sundius:
   The DFT force field of acetate ion – difficulties encountered with the SQM approach
   Proceedings of XVIIIth International Conference on Raman spectroscopy, p.113, 2002

3. G. Keresztury, T. Sundius, **K. István**:
   Applicability of the SQM force field to the vibrational spectra of sodium acetate
   Proceedings of the XXXVII Annual Conference of the Finnish Physical Society, p. 71, 2003

4. G. Keresztury, S. Holly, **K. István**, T. Sundius, T. Lóránd:
   Analysis of vibrational spectra of some new E- and Z-4-arylidene-3-isochromanones
   Part2. Isomers and conformers of the 2'-pyrrolyl and 2'-nitrophenyl derivatives
   J. Biochem. Bioph. Meth., **61,** 107-118, 2004 IF: 1.611

5. G. Keresztury, **K. István**, T. Sundius:
   Applicability of the SQM force field method to the vibrational spectra of charged molecules – I. sodium acetate
   J. Phys. Chem.,(submitted)

Oral and poster presentations:

1. **K. István**, G. Keresztury, O. Berkesi, T. Sundius:
   Vibrational spectra and force field of acetate ion – difficulties encountered with the SQM approach
   VIIIth International Conference on Raman Spectroscopy, Budapest, Hungary, 25-30 Aug., 2002; Poster No.: 13/M

2. **K. István**, G. Keresztury, O. Berkesi, T. Sundius:
   Vibrational spectra and force field of acetate ion – difficulties encountered with the SQM approach
   XXVIth European Congress on Molecular Spectroscopy, Villeneuve d'Ascq, France, 1-6 Sept., 2002; Poster No.: P14.19

3. G. Keresztury, M. Rogojerov, **K. István**, and B. Jordanov:
   Advances in determination of transition moment directions by IR-LD spectroscopy and computational methods
   XXVIth European Congress on Molecular Spectroscopy, Villeneuve d'Ascq, France, 1-6 Sept., 2002; Poster No.: P14.18

4. J. Halász, **K. István**, I. Ráthonyi, Z. Kónya:
   Identification of active centers of Sb-Sn-V oxide catalysts by FT-IR and Raman spectroscopy
   XXVIth European Congress on Molecular Spectroscopy, Villeneuve d'Ascq, France, 1-6 Sept., 2002

5. G. Keresztury, T. Sundius, **K. István**:
   Applicability of the SQM force field to the vibrational spectra of sodium acetate
   XXXVII Annual Conference of the Finnish Physical Society, Helsinki, Finnland, 20-22 March, 2003

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