Designation: E 2106 – 00

Standard Practice for General Techniques of Liquid Chromatography-Infrared (LC/IR) and Size Exclusion Chromatography-Infrared (SEC/IR) Analyses[1]

This standard is issued under the fixed designation E 2106; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This practice covers techniques that are of general use in qualitatively analyzing multicomponent samples by using a combination of liquid chromatography (LC) or size exclusion chromatography (SEC) with infrared (IR) spectrometric techniques. The sample mixture is separated into fractions by the chromatographic separation. These fractions are subsequently analyzed by an IR spectroscopic method.

1.2 Three different types of LC/IR techniques have been used to analyze samples **(1,2)**.[2] These consist of eluent trapping (see Practices E 334), flowcell and direct deposition. These are presented in the order that they were first used.

1.3 The values stated in SI units are to be regarded as standard.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*

E 131 Terminology Relating to Molecular Spectroscopy[3]

E 168 Practices for General Techniques of Infrared Quantitative Analysis[3]

E 334 Practices for General Techniques of Infrared Microanalysis[3]

E 355 Practices for Gas Chromatography Terms and Relationships[3]

E 932 Practice for Describing and Measuring Performance of Dispersive Infrared Spectrometers[3]

E 1252 Practice for General Techniques for Qualitative Infrared Analysis[3]

E 1421 Practice for Describing and Measuring Performance of Fourier Transform Infrared (FT-IR) Spectrometers: Level Zero and Level One Tests[3]

3. Terminology

3.1 *Definitions*—For definitions of terms and symbols, refer to Terminology E 131.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *hit quality index (HQI), n*—the comparison of infrared spectroscopic data against a database of reference spectra of known compounds is often employed to assist in the determination of the evolved gas chemical identity. Search algorithms generate a listing of reference compounds from the database that are spectroscopically similar to the evolved gas spectrum. These reference compounds are ranked with regard to a measurement of the comparative fit of the reference spectral data to that of the spectrum of the evolved gas. This ranking is referred to as the hit quality index (HQI).

4. Significance and Use

4.1 This practice provides general guidelines for the practice of liquid chromatography or size exclusion chromatography coupled with infrared spectrometric detection and analysis (LC/IR, SEC/IR). This practice assumes that the chromatography involved is adequate to resolve a sample into discrete fractions. It is not the intention of this practice to instruct the user on how to perform liquid or size exclusion chromatography (LC or SEC).

5. General LC/IR Techniques

5.1 Three different LC/IR techniques have been used to analyze samples. These consist of eluent trapping, flowcell and direct deposition. These are presented in the order that they were first developed. Infrared detection for any of these techniques can be provided by IR monochromators, IR filter spectrometers and Fourier transform infrared spectrometers (FT-IR). These detectors yield either single absorption band or total infrared spectrum detection modes. Detection mode is dependent upon the type of IR detector employed and the acquisition time required by the LC or SEC experiment.

5.2 *Eluent Trapping Techniques*—Eluent trapping techniques, such as stopped flow and fraction collection, are the simple means for obtaining LC/IR data. In these techniques, the eluting sample is collected from the chromatograph in discrete aliquots. These aliquots are then analyzed with the appropriate sampling accessory in an infrared spectrometer. In utilizing such techniques, it is essential that a suitable LC

[1] This practice is under the jurisdiction of ASTM Committee E13 on Molecular Spectroscopy and is the direct responsibility of Subcommittee E13.03 on Infrared Spectroscopy.

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[2] The boldface numbers in parentheses refer to the list of references at the end of this standard.

[3] *Annual Book of ASTM Standards*, Vol 03.06.

detector, such as refractive index or UV/VIS, be employed to allow definition of component elution. Since the analyte of interest is trapped physically, the spectrum can be recorded using a long integration or scan coaddition time to improve the signal-to-noise ratio (SNR). Generally, the stopped flow technique requires the use of a flow cell and the IR spectrum acquired contains both analyte and mobile phase spectral features. The fraction collection mode permits examination of the eluent as a solution of analyte and mobile phase or, with proper solvent removal, the analyte alone (provided that the analyte is nonvolatile). As such, the fraction collection mode would require either a liquid cell for solutions or a solid substrate, that is, KBr window for transmission, first surface mirror for reflection-absorption or powdered KBr for diffuse reflection measurements.

5.3 *Flowcell Detection*—With flowcell detection, the LC eluent is monitored continuously in the timeframe of the chromatography (real-time) by the IR spectrometer with the use of specially designed liquid cells **(3-9)**. Liquid cells are designed to minimize dead volume and analyte mixing, to conserve chromatographic resolution, and achieve maximum optical interaction of the eluent with the infrared radiation. As the effluent is a condensed phase, several cell types have been devised to accommodate most experimental approaches for IR spectrometry, that is, transmission, reflection-absorption and attenuated total reflection **(7)**. The flowcell technique typically yields submicrogram detection limits for most analytes **(1)**. Typically, flowcells are mounted within the sample compartment of the spectrometer and use beam condensation optics to direct the IR beam into and out of the small volume of the cell. It is important to employ a mobile phase having low or preferably no infrared absorptions in the analytically important spectral regions for the analytes of interest. As such, the choice of mobile phase may constrain the liquid chromatographic separation. Generally, this limits the chromatographic separation to a normal phase type where nonpolar solvents like chloroform and carbon tetrachloride have sufficient solvent strength to elute components and have low infrared absorption. In contrast, flowcell detection of reversed phase separations involving aqueous mobile phases are essentially precluded as strong absorption by water occurs across the mid-infrared spectrum. If flowcell detection of reversed phase separation is to be attenuated, removal of the analytes from the aqueous mobile phase via extraction into an infrared transmissive solvent is suggested **(9)**.

5.3.1 The rapidity with which spectra must be recorded during a liquid chromatographic separation typically requires a Fourier-transform infrared (FT-IR) spectrometer to capture the complete infrared spectrum. Such instruments include a computer that is capable of storing the large amount of spectroscopic data generated for subsequent evaluation. Conversely, monochromators and filter infrared spectrometers permit the monitoring of a selected absorbance band, for example, 1730 cm^{-1} for carbonyl functional groups. Data acquisition for these devices is similar to that for a typical LC detector.

5.3.2 The transfer line from the LC column to the flowcell must be made of inert, nonporous material. This normally is PTFE, PEEK or stainless steel tubing. The volume, internal diameter, and connections of the transfer line are optimized to reduce dead volume and mixing that can degrade the chromatographic separation. When performing separations at elevated temperatures, the transfer line and flowcell may require controlled heating to maintain temperatures of the eluent.

5.3.3 The flowcell is made of IR transmissive window materials to give maximum optical throughput to and from the effluent chamber. Proper selection of window material is necessary to ensure chemical inertness and IR transmissivity. The cell design and volume must maintain chromatographic resolution while maximizing optical interaction with the eluent via transmission, reflection-absorption or attentuated total reflection modes. Flowcells are typically optimized so that the sampling volume accommodates the corresponding eluent volume of a sharp chromatographic peak at the peak's full width at half height (FWHH). Typically, this volume is matched to the scale of the liquid chromatography, that is, 10 µL for analytical scale and larger volume separations and less than 10 µL for microbore separations.

5.3.3.1 The optimum infrared transmission across the full mid-infrared spectrum is obtained by using potassium bromide windows; however, this material is susceptible to damage by water and cold flows under mechanical force. As the flowcell is used, small amounts of water will etch the window surfaces, and the optical throughput of the windows will drop. Eventually, these windows will have to be changed. Users who expect to analyze mixtures containing water should consider using windows made of a water-resistant material such as zinc selenide (ZnSe). IR windows of high refractive index like ZnSe and zinc sulfide (ZnS) will result in a noticeable drop in infrared transmission due to the optical properties, that is, reflectivity, of such materials. Additionally, high refractive index materials may cause fringing, that is, create an optical interference pattern in the baseline of the IR spectrum.

NOTE 1—Fringing is due to multiple reflection optical paths created when windows are placed as parallel plates separated by a discrete pathlength. These reflection optical paths permit light, which is retarded to a greater extent than light from the transmitted optical path, to reach the detector. This reflection optical path light is out of phase with the transmitted optical path light and yields interferences fringes in the resultant spectrum. Fringing may be reduced by making the windows nonparallel or by placing the cell slightly askew, that is, 5–15°, in the optical beam of the spectrometer. Please refer to Practices E 168 for additional information on fringing effects.

5.3.3.2 The optical energy throughput of the flowcell should be periodically monitored, since this is a good indicator of the overall condition of the LC/IR interface. If a Fourier transform spectrometer is used, it is recommended that records be kept of the interferogram signal strength, single-beam energy response, and the ratio of two successive single-beam curves (as appropriate to the instrument used). For more information on such tests, refer to Practice E 1421. These tests will also reveal when a mercury cadmium telluride (MCT) detector is performing poorly due to loss of the Dewar vacuum and consequent buildup of ice on the detector face. As noted further in this text, an MCT detector is commonly used with these experiments as they provide greater detectivity and fast data acquisition times.

5.3.3.3 Care must be taken to stabilize or, preferably, remove interfering spectral features resulting from atmospheric

absorptions in the optical beam path of the spectrometer. Best results will be obtained by purging the complete optical path with dry nitrogen gas. Alternatively, dry air can be used for the purge gas, but has interferences in the regions of carbon dioxide IR absorption (2500 to 2200 cm^{-1} and 668 cm^{-1}). Commercially-available air scrubbers that remove water vapor and carbon dioxide also provide adequate purging of the spectrometer. In some instruments, the beam path is sealed in the presence of a desiccant, but interferences from both carbon dioxide and water vapor (1900 to 1400 cm^{-1}) may still be found. In all cases, the instrument atmosphere must be stabilized before data collection commences. Atmospheric stability inside the instrument can be judged by recording the single-beam energy response and the ratio of two successive single-beam spectra.

5.4 *Direct Deposition LC/IR*—Initial attempts at direct deposition LC/IR employed eluent deposition onto powdered KC1 **(10)**. After evaporation of the mobile phase, the analysis of analytes was conducted by diffuse reflection. More recently, the direct deposition LC/IR technique is accomplished by deposition of the eluent onto a flat, moving surface to allow analysis by transmission or reflection-absorption **(11,12)**. In these methods, the eluent is passed through a nebulizer to atomize the mobile phase, the aerosol is passed through a heated transfer zone to evaporate the mobile phase and the residue is deposited onto an appropriate optical substrate. This allows for these methods to detect as low as subnanogram amounts of material. By capturing the eluent onto a substrate, the components of the sample are effectively trapped. It is possible, therefore, to analyze the chromatographic distribution of analytes after the LC/IR experiment as well as to perform analyses in real-time.

5.4.1 For transmission spectra, the eluent is deposited directly onto an infrared transmissive plate maintained at a temperature sufficient to permit further evaporation of the mobile phase **(11)**. Infrared spectra are then obtained via an infrared transmission method.

5.4.2 For reflection absorption measurements, the eluent is deposited upon a front surface mirror. The infrared beam is then transmitted through the analyte, reflected off the mirror surface and transmitted back through the analyte. A modification of this method has been introduced where the eluent is deposited upon a thin germanium wafer. The back surface of this wafer is vapor coated with aluminum to yield a reflective surface **(12)**. As germanium is IR transmissive, the beam passes through the deposited analyte twice and, depending upon the angle of incidence and reflection, yields an approximate doubling of the pathlength. The advantage of this approach over that of a first surface mirror is to reduce spurious optical effects such as specular reflection which may occur as light passes through the spotted analyte.

5.4.3 Direct deposition techniques provide the advantage of post-run spectral data acquisition and possibly, decoupling the chromatographic separation from the spectrometry. Through extended co-addition of spectra, the signal-to-noise ratio (SNR) of spectral results is improved over that obtained during real-time data acquisition. It must be noted that slow sublimation of the analyte and recrystallization may occur with direct deposition techniques. It is prudent to initially obtain the spectral data with a short co-addition time to create reference data to ensure the integrity of spectra obtained with longer co-addition times after the chromatographic separation is complete.

6. Significant Parameters for LC/IR

6.1 The instrumentation used to conduct the LC/IR experiment should be properly recorded within prescribed standard operating procedures (SOPs) or laboratory notebooks as necessary to meet requirements for specific laboratory practices. If the equipment is commercially available, the manufacturers' names and model numbers for the complete LC/IR system, or the individual components, should be recorded. Additionally, various instrumental and software parameters are listed and discussed in 6.2-6.4.5 Any modifications made to a commercial instrument must be clearly noted.

6.2 *Instrumental Parameters (IR)*:

6.2.1 *Detectors*—Due to low optical throughput, most LC/IR systems typically employ MCT narrow band photoconductive detectors. It is important that the detector element be properly filled with the image of the analyte spot or image of the exit aperture of the interface to achieve the highest signal-to-noise. Additionally, care must taken to ensure the MCT detector is not operated in a light saturating condition so as to maintain linearity of the signal response. Alternatively, deuterated triglycine sulfate (DTGS) detectors may be appropriate as these extend the spectral range to 400 wavenumbers. These are slow detectors, however, and are used typically with direct deposition and discrete fraction collection methods where spectral acquisition times are not critical.

6.2.2 *Flowcell Interface*—A complete description of the flowcell interface including optical type, optical pathlength, volume, temperature and material type should be recorded.

6.2.3 *Deposition Conditions*—For direct deposition LC/IR, the temperature of the nebulizer, evaporation chamber and deposition surface should be recorded. Type of nebulization gas, its flowrate and temperature, as well as the motion of the deposition surface should be noted. Spot size of the eluent deposited is directly determined by the diameter of the restriction end (nebulizer) and the distance separating the restriction end from the deposition surface. These should be recorded.

6.3 *Instrumental Parameters (LC)*—The success of the LC/IR experiment is dependent on good chromatographic practices. It is not the purpose of this document to discuss those practices in detail, but for convenience, a list of some LC parameters of key importance to be noted is given.

6.3.1 *Chromatographic Column*—The length and internal diameter of the column including the type of stationary phase employed must be noted.

6.3.2 *Mobile Phase*—The type of mobile phase used should be noted. If binary or ternary solvent systems are employed, the gradient profile should be specified in detail and include any initial delay or final hold time.

6.3.3 *Injection*—The injection volume, solvent matrix, sample concentration, if appropriate, are critical parameters that must be recorded.

6.3.4 *Other Chromatographic Detectors*—If a chromatographic detector is employed in addition to the IR spectrometer, then the following information should be listed: type of detector and whether the detector is serial or in parallel (in which case, the eluent split ratio should be specified).

6.4 *Software Parameters*:

6.4.1 *Apodization Function*—For Fourier transform infrared spectrometers, it is recommended that an apodization function be applied to the interferograms before computation of spectral data. Suitable apodization functions include triangular, Beer-Norton medium, Happ-Genzel, and cosine function.

6.4.2 *Spectral Resolution*—For Fourier transform spectrometers, a compromise between the signal to noise ratio (SNR) of a spectrum and its information content leads to an optimum resolution for LC/IR spectra of 8 cm^{-1} if recorded in real-time. If spectra are acquired from trapped samples, higher spectral resolution may be appropriate.

NOTE 2—Most conventional LC/IR instruments are optimized to record a spectrum of 8 cm^{-1} resolution in approximately one second. This allows for adequate sampling of the spectral data as a chromatographic peak flows through the flowcell; thus, the optimal SNR is obtained for spectral data with minimal loss of chromatographic resolution and sufficient chromatographic peak definition.

When examining samples by direct deposition LC/IR, real-time spectra are optimally collected at 8 cm^{-1} resolution for the above reason. When employing post-run signal averaging, however, data can be collected at a greater resolution to increase the information content of the spectra. The appropriate spectral resolution is often that which matches the resolution of available spectral libraries suitable for condensed phase samples.

6.4.3 *Signal Averaging*—During real-time data acquisition, it is advantageous to co-add several scans/time increment, generally, a 1–3 s time frame, to improve the SNR of the result. The actual number of co-additions depends on the instrument's optimal scanning speed and spectral resolution. Typical instrument operation would permit co-addition of four to ten scans during each time increment, that is, a discrete infrared spectrum is stored every second. More extensive spectral averaging can be performed during post-run spectral collection or data manipulation; thus, during real-time data collection, the SNR improvement is limited to the total elution time of an analyte.

6.4.4 *Data Storage Threshold*—This function must be recorded, if used.

6.4.5 *Additional Processing*—If any smoothing functions, baseline correction algorithms or spectral subtractions are applied to the spectral information, these must be reported. It should be pointed out that most commercial LC/IR instruments give the operator only a limited control over these functions, which may be operating automatically. The operator should investigate as to whether the instrument software does include such operations.

7. Software Treatment of Infrared Data

7.1 Infrared spectral information acquired during the liquid chromatographic separation can be manipulated in either the real-time or post-separation domain to yield chemical information. Some software programs permit display of chromatograms using spectral information, that is, Gram-Schmidt reconstruction (GSR) **(10,11)**. total IR absorbance and specific IR absorption bands, in real-time. Additionally, these software programs may permit data manipulation on-the-fly, that is, co-addition, smoothing, spectral subtraction, and spectral searching, during the chromatographic separation. This section describes these spectral data manipulation activities.

7.1.1 *Gram-Schmidt Reconstruction (GSR)* **(10,11)**—As each interferogram is acquired during the chromatographic separation, a method for tracking total IR intensity, called the Gram-Schmidt reconstruction (GSR) **(10,11)**, quickly determines the information content of the interferogram. In this method, a set of interferograms is recorded before sample injection and individual interferograms are recorded during the separation. The initial set of interferograms is used to create a series of reference basis vectors that represent the chromatographic baseline profile. During the separation, each subsequent interferogram generates a similar vector. Comparison of these individual vectors against the reference set is performed via orthogonalization to give a measure of the presence, or absence, of analytes eluting from the chromatographic column and their relative concentration. The resulting plot of vector intensity versus time indicates how the total infrared intensity changes during the separation. This is called the Gram-Schmidt reconstructed (GSR) chromatogram and is similar in appearance to the response from a refractive index or UV/VIS detector. This chromatogram normally is displayed on the computer screen.

7.2 *Data Storage Threshold*—With older LC/IR systems, the large number of spectra recorded during a typical LC/IR experiment would be more than could be stored with the available computer. Because of this, the Gram-Schmidt reconstructed chromatogram was monitored and, when a GSR intensity change exceeded a preset threshold value, the spectra were acquired and stored. It was possible that if a minor component was not detected during elution, no spectral data were stored for it. With current data storage capabilities, data storage threshold is rarely used as the typical LC/IR file sizes are easily accommodated.

7.3 *Functional Group Chromatograms*—As each interferogram is acquired, it may be processed into a spectrum in real-time. In this case, a useful operation is to integrate selected regions of the spectrum immediately and to present the integrated absorbance values to the display along with the Gram-Schmidt reconstructed (GSR) chromatogram. Commonly selected infrared spectral regions with the typically corresponding functional groups are as follows:

Unsaturated/Aromatic C-H Stretch: 3150 to 3000 cm^{-1}
Saturated C-H Stretch: 3000 to 2850 cm^{-1}
C=O Stretch: 1800 to 1650 cm^{-1}
C-O Stretch: 1300 to 1000 cm^{-1}

7.3.1 It should be noted that these absorption band assignments do not correspond to all possible functional groups that may absorb in these, or other, spectral regions. Absorption band assignments should be verified with appropriate reference materials.

7.4 *Spectral Searching*—The general purpose of the LC/IR experiment is to attempt to identify or classify the chemical nature of the species that are separated chromatographically. To do so, absorbance spectra of each fraction are generated, either automatically or with operator interaction. Spectra are compared individually, using one or several software search algorithms, to a library or database of reference spectra. These

databases typically reside in a digitized format on the computer disk. The spectral search results yield a list of potential identities that match the sample spectrum most closely. These identities are ranked in order of their match quality.

7.4.1 It should be stressed that the results of a spectral search cannot be relied upon to obtain the correct chemical identity. Potential problems that the user should consider include mislabeled library entries, poor spectral match quality, differences between acquired spectra and those of the library due to experimental conditions, absence of the relevant reference in the database, low signal to noise ratio of the measured spectrum, and similarity between the reference spectra of members of a homologous series. The analyst should always verify the search results by visually comparing the spectrum of the best matches to the analyte spectrum.

7.4.2 An important parameter to be considered for spectral searching is the search algorithm. Some algorithms, such as the Euclidean algorithm, match the spectrum point by point with each reference spectrum in the database. This method takes into consideration the relative intensity, shape, and frequency of each spectral feature, but places the heaviest importance on strong, broad features. If the derivative of the analyte spectrum is compared to the derivative of each reference spectrum, however, emphasis is placed upon the absorption band frequencies of sharp bands. Any shifts in absorption band frequencies between the spectra of the sample and reference typically lead to poor matches when using the derivative algorithm. The selection of search algorithms to employ should consider the sharpness of spectral features and similarity in spectral acquisition method, that is, IR transmittance, reflectance or diffuse reflectance, between the analyte spectrum and database reference spectra. As several search algorithms are available, it is important to understand how each one operates upon the spectral data to obtain effective use of spectral searching. The results of a spectral search often are placed in a table where reference compounds whose spectra closely fit that of the analyte are ranked with regard to the hit quality index (HQI).

7.4.3 It is common practice to generate reference databases containing spectra of compounds expected for the analyses commonly performed by the laboratory. These spectra are often obtained under similar conditions to those used for the LC/IR experiment. Libraries of spectral data are also available from commercial sources. Condensed phase LC/IR data are best compared to reference spectra recorded for compounds in their condensed phases.

7.5 *Spectral Subtraction*—To enhance spectral information, the absorbance spectrum of a reference material may be subtracted from the absorbance spectrum of a mixture. With the spectral contributions of the reference material removed, the resultant spectrum is more representative of the true eluite chemical identity. Mathematical subtraction of spectra may be used to improve the quality of analyte spectra and to resolve analytes eluting as overlapping chromatographic peaks. For example, spectral features due to purge instability and mobile phase(s) may be removed through subtraction of atmospheric or mobile phase reference spectra from the analyte spectrum. To resolve species in an overlapping chromatographic peak, spectra obtained from the leading and trailing edges of the chromatographic peak are subtracted from each other to yield purer spectral information of the leading component and the trailing component.

7.5.1 When performing an LC/IR experiment, often it is possible to generate the spectrum of the mobile phase for subtraction purposes by examining the spectra collected close to the elution of the eluite peak; however, this activity is precluded if the data storage threshold algorithm (see 7.2) is used, as the absorbance spectra of the chromatographic baseline would not be stored.

7.5.2 If the absorbance spectra generated during the LC/IR experiment contain significant spectral interferences from atmospheric water vapor or carbon dioxide, then a reference spectrum of one or both of these contaminants may be subtracted from each analyte spectrum. Typically, these interferences will be observed also in the spectra of the chromatographic baseline; thus, an atmospheric reference spectrum may be generated for subtraction that is close in the chromatography to that of the analyte spectrum. Also, it is possible to have the computer automatically remove spectral contributions from atmospheric contaminants if appropriate reference spectra are available.

7.5.3 Occasionally, an analyte peak of interest is close to, or overlapping with, the chromatographic peak due to the injection solvent. This occurs at the elution of an unretained eluite and defines the column's chromatographic void volume. Spectral subtraction typically is employed to remove spectral contributions from the injection solvent in the spectrum of the analyte chromatographic peak.

7.5.4 On occasion, two eluites will elute at similar times, so that they overlap each other and yield a multicomponent chromatographic peak. Often, it is possible to generate spectra at the leading and trailing edges of this chromatographic peak, which reveal differential concentrations of the individual eluites. Depending on this differential, these spectra may represent the two pure compounds or mixtures of both. In the latter case, subtraction of the spectrum of the trailing edge from that of the leading edge will yield a purer spectrum of the leading edge eluite. Subtraction of the leading edge spectrum from that of the trailing edge will yield a purer spectrum of the trailing edge eluite. This spectral subtraction method also is appropriate for chromatographic peaks that have more than two components if those components have differential concentration profiles through the chromatographic peak. Use of this method, however, becomes more complex as multiple reference spectra from the chromatographic peak must be removed through multiple subtraction steps.

8. Standard Samples

8.1 In addition to validations of the liquid chromatographic system and the IR spectrometer (Practice E 1421), a validation test separation should be used on a regular basis to evaluate and document the LC/IR instrument response. These validations would also indicate when problems have developed in the IR spectrometer, the liquid chromatograph or the LC/IR interface. In the LC/IR validation method, the parameters discussed in Section 6 are established and must be reproduced exactly each time the validation is performed. Depending on the type of LC/IR validation being performed, a uniform validation

sample mixture would be created with the appropriate components and component concentrations. The validation procedure should involve calculation of the signal-to-noise ratio of the LC/IR spectra of each validation mixture component, as well as, that of the chromatographic baseline. During this procedure the SNR may be calculated in terms of either peak-to-peak noise or root-mean-square noise, but the method used to generate the SNR must be specified. The user also is referred to the instrument documentation supplied by the equipment manufacturer.

9. Sampling Criteria

9.1 The sampling criteria for the various LC/IR techniques depend on the analytical application. The advantages and disadvantages are discussed for each sampling technique (see Section 5). These must be taken into consideration when determining the best method for combined separation and identification for a particular sample analysis or application.

10. Qualitative Information

10.1 In the LC/IR experiment, the infrared spectrometer acts as a chromatographic detector. In real-time chromatographic detection, an infrared monochromator or filter spectrometer provides an absorbance response versus time for a discrete frequency range. In contrast, since an FT-IR spectrometer acquires the full IR spectrum at discrete time intervals throughout the chromatographic separation, several different chromatographic representations may be obtained. The Gram-Schmidt reconstructed chromatogram (see 7.1), therefore, which resembles a refractive index chromatogram, can be used for location of spectroscopic data files along the chromatographic time domain. Infrared functional group chromatograms (see 7.3) selectively detect eluting compounds with specific functional groups.

10.2 Spectral identification by reference spectral database searching is recommended for compound identification in the LC/IR experiment. Best results will be obtained when the spectra in the reference database are acquired in the same fashion as that of the eluite. For example, if the eluite spectrum is acquired from a deposition LC/IR interface in the reflection-absorption mode, then spectra of reference compounds should be acquired in the same manner. In this manner, influences on the spectral information related to instrumentation and the spectroscopic method are minimized in the spectral searching process and yield superior search results. If reference databases of this type cannot be created, searching against condensed phase reference databases, either commercially available or user-generated, may yield acceptable spectral searching. It should be noted that influences on reference spectra due to the spectroscopic method, for example, transmission, reflection-absorption, diffuse reflection, use of nujol, etc., will affect the quality of the spectral match and may lead to erroneous results.

10.3 For a more reliable validation of the identity of a chromatographic peak, a suitable reference compound should be examined by LC/IR using identical conditions. In this procedure, it is important that the chromatographic retention times be matched, as well as the spectra of the unknown and the reference compound. Alternatively, other analytical techniques can be used to verify the identity of the unknown material once it is isolated.

11. Quantitative Information

11.1 Reasonably accurate quantitative information can be obtained by LC/IR methods. Measured values within 10 % of the actual quantities are attainable with careful reproduction of experimental parameters. In order to obtain quantitative results, comparison should be made to reference standard mixtures examined under identical conditions. The integrated chromatographic responses of reference standards are used to calibrate the chromatographic method. For general aspects of quantitative analysis performed with infrared spectrometry, essential references are found in Practices E 168 and the chapter in Willis, *et al*., **(15)**.

11.2 Similarly, the amount of each component present in the mixture can be ascertained from a LC chromatographic trace generated from detectors other than an IR detector. In this case, the quantity of a component can be calculated from its integrated peak area and compared to the integrated response curve of reference standard samples used to calibrate the chromatographic separation.

11.3 Quantification methods corresponding to standard techniques should be performed in accordance with Practices E 168. When increasing sample quantities are injected, deviations from Beer's Law may occur as absorbance values exceed 1 au. While detection limits vary between the various LC/IR methods, the linear ranges of each method are approximately equal, at between two and three orders of magnitude. It should be noted that the direct deposition techniques are designed for low detection limits with small sample quantities.

11.4 Integration across the appropriate spectral region or measurement of peak height from the Gram-Schmidt reconstructed chromatogram can also be used for quantification. These methods can yield values of good precision and accuracy and are similar to quantification of chromatographic data via other detection modes.

12. Record of the Data

12.1 It is recommended that the unprocessed spectroscopic data, for example, interferograms for FT spectrometers, be stored digitally in addition to spectra.

12.2 *Storage of Raw Data*—Present LC/IR technology is based on rapid scanning FT-IR spectrometers and methods. These instruments permit storage of the raw data as interferograms and spectra. The LC/IR data should be stored on a magnetic or optical media such as a hard drive, tape backup or compact disk system capable of read-write operations. In this case, the relevant background single-beam spectrum, vectors calculated for Gram-Schmidt reconstruct, and the Functional Group and Gram-Schmidt chromatograms should be stored with the data. These extra files may be required by the software to permit analysis of the raw data files. It is important to store an information file that contains not only the instrumental parameters, as in Section 6, but also a record of the sample identity, solvent matrix, and any treatment carried out on the sample.

12.3 *Storage of Infrared Spectra*—In addition to the raw data, the LC/IR experiment will result in a series of infrared spectra corresponding to some, or all of the chromatographic

peaks. These spectra occupy a substantially lower amount of storage space than the raw data, and can be saved easily on a more convenient medium, such as a floppy disk. Again, all instrument parameters, sample identity information, etc., should be stored with the spectral data set. These spectra are then readily available for subsequent plotting, searching, or other use. Also, it may be useful to store them with the raw data, in accordance with 12.1.

12.4 *Spectral Interchange*—A universally compatible format for digital storage of data, JCAMP-DX, has been developed **(16)**. This protocol generally is useful for transferring individual spectra between instruments of different manufacturers. It is not likely that the large raw data files, such as LC/IR data sets will be transferable in this way due to limitations of storage space and RS232 transfer speed. All spectrometer manufacturers have provided, or are in the process of producing, provisions for converting digitally stored data in their own software formats to that of the universally transportable JCAMP-DX. It is recommended that data be handled in accordance with each manufacturer's specification that would allow for conversion into JCAMP-DX for maximum versatility.

12.5 *Information to Appear With the Spectrum*—The sample identification and source of the sample, if known, should appear with the spectrum. The solvent matrix and any sample preparation should also be noted. An indication of the quantity of sample should be given, together with the pathlength, volume, shape, and temperature of the cell or sample substrate. The sampling method should also be given. If flowcell LC/IR techniques are used, the half width of the LC peak, the mobile phase flow rate, the full width at half height, the analyte retention time and the data acquisition time should be stated. The make and model of the spectrometer should be recorded, as well as the model name or number of the LC/IR interface. For spectra measured on dispersive spectrometers all changes of gratings and filters should be recorded, together with the wavenumbers at which they occur. The date on which the spectrum was measured should also be given.

13. Keywords

13.1 infrared spectroscopy; LC/IR; liquid chromatography; SEC/IR

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