Indirect Detection Methods: Looking for What Is Not There

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Introduction

Traditionally, chemical instrumentation is designed with the species of interest in mind. We learn to use an infrared photometer to detect gaseous HCl but not for detecting gaseous H_2. Because we have a species in mind, the instrument can be fine-tuned for optimum performance. What happens when the instrument (transducer) does not respond to the species of interest (the analyte) because it lacks certain physical or chemical properties? One can often operate the instrument in the indirect detection mode, which makes it possible to detect species that do not themselves provide a response.

Chromatography falls into one of these unusual situations. Normally, chromatography is used to separate different species on the basis of their retention on the column. There is always a mobile phase that carries the sample components as they migrate down the column. The presence of the mobile phase forms the basis for the instrumental response. What one can do is to select a mobile phase that possesses the physical or chemical properties to which the instrument responds. This is depicted in Figure 1. The detector responds to the mobile phase or one of its components, as indicated by the open circles. There is then at all times a large background signal at the detector. When the species of interest, denoted by the solid circles in Figure 1, are eluted off the column, some mode of displacement occurs. Examples of displacement mechanisms are the conservation of charge or the conservation of volume. When this occurs, there will be less of the mobile-phase component present at the detector. A smaller signal will be observed. Nonetheless, this allows detection of the analyte, and the normal functions of quantitation (peak area) and identification (peak position) are retained. The response is therefore an indirect one and is a result of the *absence* of the mobile-phase component rather than the *presence* of the analyte. This is the key feature of indirect detection methods.

There are several related detection schemes in chromatography that are easily confused with indirect detection. Vacancy chromatography[1] produces positive or negative peaks for the analytes. However, as discussed in section 3 below, the instrument in fact responds to the analyte directly. Quenching of fluorescence by the analyte has been used for detecting nonfluorescent species.[2] Fluorescence quenching sometimes also refers (incorrectly) to absorption by the analyte at either the excitation or the emission wavelength of the fluorophore. In both cases, the response of the fluorophore (rather than its concentration) is altered by a specific interaction. The amperometric response of electrochemical detectors is surface-area dependent. This gives rise to pulsed amperometric detection[3] that produces a decrease in signal when the (electroinactive) analytes are adsorbed onto the electrode surface. There, the transducer is affected by the presence of the analyte and not the absence of the mobile-phase component. So, one has to be careful not to confuse the observation of negative signals (decreases in response) as an indirect measurment. In fact, as will be shown later, indirect detection can actually involve positive signals (increases in response).

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Reasons for Indirect Detection

Apart from the intellectual curiosity of using an instrument to measure something that does not show a response, there are good reasons to develop indirect detection schemes. First, indirect detectors are universal, i.e., there is little requirement as to the exact nature of the analyte. Naturally, it has to be different from the mobile phase or the added component in terms of the response at the detector and it has to participate in the particular displacement mode. In other words, even analytes that normally show a response at that detector will give an indirect signal, as long as the response is different (per mole, per volume, or per equivalent) for the mobile-phase component. A corollary is that the more unique the mobile-phase component (more selective response), the more broadly applicable the indirect detector becomes. Universal detectors are needed in the development of analytical methods when chemical derivatization of the analyte is difficult because of limited quantities or because of the lack of active functional groups, e.g., alkanes.

Second, there is the possibility of sensitivity transfer. Some of the high-sensitivity detectors are also very selective. It will be useful to broaden the applicability of these detectors by implementing indirect detection. By proper design, it is possible to take advantage of the available sensitivity. In general, it is difficult to achieve the same low limit of detection (LOD) for indirect detection compared to direct detection because noise is usually larger in the presence of a large background signal. Still, coming within 1 or 2 orders of magnitude of the LOD of high-sensitivity detectors that otherwise

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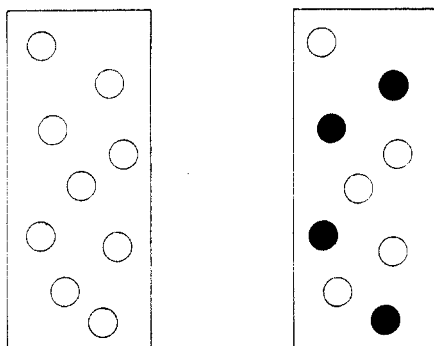

Figure 1. General scheme for indirect detection. Left: the mobile phase contains a component that provides the actual response (open circles). Right: when analytes (solid circles) reach the detector, displacement results in a decrease in the background signal, i.e., a negative peak.

do not show a response is very useful.

Third, quantitation is easier with indirect detection. Not only is tedious sample manipulation (e.g., chemical derivatization) avoided, but the instrumentation is also simplified. One needs just one detector for almost all situations. One can select the appropriate mobile-phase component to fit the instrument. For example, a simple fixed-wavelength filter fluorometer may suffice rather than a sophisticated double-monochromator system. The response is also more predictable because it is derived from the mobile-phase component. The response should be fairly uniform, especially if the mobile-phase component is unique. That is, one can use the same calibration curve for a variety of analytes. Even if the component is not unique, it may be possible to perform quantitation without standards by using two separate mobile-phase components[4] as a universal calibration.

Fourth, indirect detection is nondestructive. This is because no chemical manipulation has been introduced. The analyte can be collected as in preparative chromatography for further analysis or for other use. This is particularly important in biotechnology, where samples are valuable and sample preservation is critical. Indirect detection also allows characterization of the analyte in its native form, such as proteins and pharmaceuticals. The only "contamination" is from the mobile-phase component, which by its nature must not interact with the analyte in order to allow detection by displacement.

Requirements for Indirect Detection

While almost all detection schemes can be made to function in the indirect mode as prescribed by Figure 1, the adaptation may not be useful at all. What is needed is a predictable response, as well as one that allows the detection of low concentrations of analyte. That is, the mechanism for displacement must be clear and unambiguous, and the operating conditions must be amenable to optimization at low analyte concentrations.

We can define a transfer ratio, TR, as the number of mobile-phase molecules displaced (or replaced) by one analyte molecule. In the simplest case, displacement is by volume. That is, in the fixed volume of the detector, to accommodate the analyte molecules, there has to be a smaller amount of the bulk mobile phase present. Another possibility is charge displacement. If the mobile-phase component is negatively charged, then negatively charged analytes will displace the mobile-phase component on a per equivalent basis. This is because there is a constant concentration of counterions in the flow stream, and local charge neutrality must be maintained. It follows that if instead the analytes are positively charged (oppositely charged as the mobile-phase component), then more of that mobile-phase component must be present. An increased (positive) response will result. Displacement can also be due to solubility modification. There is always partition between the stationary phase and the mobile phase in chromatography. The analyte can modify this partition by increasing or decreasing the solubility of the mobile-phase component in the stationary phase or in the mobile phase.[5] This then leads to an increase or a decrease in the background signal when the analyte is eluted off the column and reaches the detector.

The other major consideration is the stability of the background signal. In indirect detection, a large background is required. Background fluctuations that normally are negligible can become a serious problem. The figure of merit is the dynamic reserve, DR, viz., the ability to measure a small change on top of a large background signal. DR is essentially the ratio of the background signal to the background noise. Note that this is quite different from the dynamic range, which only refers to the high and the low concentration limits for useful operation. As will be described below, DR can range from 10 to 10^8, depending on the type of instrument used and the concentration of the fluorophore (which determines the magnitude of the background signal).

It can be shown that

$$C_{\mathrm{lim}} = C_{\mathrm{m}}/(\mathrm{DR} \times \mathrm{TR}) \qquad (1)$$

where C_{lim} is the concentration limit of detection and C_{m} is the concentration of the relevant mobile-phase component. For a given system, the more stable the background signal (larger DR), the smaller the fractional change one can detect. The more efficient the displacement process (larger TR), the larger the change is per analyte concentration. Also, the lower C_{m} is, the larger fractional change will result for a given analyte concentration. With eq 1, one can readily determine the applicability of a given detection scheme for operation in the indirect mode.

It is instructive to consider one example of a situation where indirect detection cannot be implemented. By selecting the appropriate wavelength, it is always possible to create a background absorption in the chromatographic mobile phase. In this case, C_{m} is on the order of 10 M for typical organic solvents. TR is on the order of 1 for volume to volume displacement. The DR for absorption detectors is around 10^4. This is based on having a background absorption of 1 au, where the noise level can be as low as 10^{-4} au[6]. Equation 1 tells us that C_{lim} will be on the order of 10^{-3} M. While this shows that indirect signals can be obtained, there is little analytical utility. If the analyte must be at 10^{-3} M or higher at the detector, the injected amount will likely overload the column. For such a volume-dis-

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placement situation, one therefore needs a substantially larger DR. The DR for absorption photometry cannot be readily increased. The noise level can be reduced to 10^{-5} au when the background signal is smaller, but then there is no net gain in DR. Shot-noise (photon statistics) contributions due to low light levels reaching the phototube increase exponentially as the background absorption is increased above 1 au. For example, a background of 2 au implies a 10-fold-lower intensity transmitted through the cell. Even using higher flux light sources does not help. The large absorption creates substantial heating and results in turbulence that contributes to noise.

So, successful applications of indirect measurements depend on having instruments that provide a large DR, a displacement mechanism that allows efficient transfer of sensitivity (i.e., a large TR), and situations where the mobile-phase component that is generating the response is present at low levels. A subtle point is that one cannot simply add a low-concentration absorber in the transparent mobile phase to enhance volume-displacement-type measurements. This is because volume displacement is nondiscriminating, and TR will be proportional to C_m, resulting in no net gain in detectability.

Indirect Detection Schemes

We will discuss below several examples of indirect detection schemes in liquid chromatography (LC). Although there are also analogous schemes in gas chromatography (GC), such as the ultrasound detector[7] and the thermal conductivity detector, they are not included because detection is usually less of a problem in GC.

1. Refractive Index (RI). All RI detectors for LC are based on sensing the difference in RI between the mobile phase and the analyte. There is an implicit volume displacement as described by Figure 1. So, the RI detector is in fact an indirect detector. In the context of eq 1, C_m is large and TR is 1 (per volume). So, the only reason one achieves reasonable detection power is because DR is quite large. The noise level is around 10^{-8} RI units and 10^{-9} RI units for commercial and for state-of-the-art systems,[8,9] respectively. The same noise level is maintained regardless of the background (RI of the mobile phase) since most arrangements use mechanical compensation for setting the null point. Since the difference between the analyte RI and the mobile phase RI is in the range of 0.1–0.2 RI units, the DR is in the 10^7–10^8 range. The limit is determined by temperature variations in the mobile phase, since maintaining noise at 10^{-9} RI units requires a stability of 10^{-4} °C.

The sensitivity of the RI detector depends strongly on the analyte. The RI difference relative to the mobile phase can be 0.01, 0.1, or even 1.0 (near absorption bands) units. Both positive and negative peaks can be obtained. This is why RI is frequently incorrectly classified as a direct detector—that the response mainly depends on the RI of the analyte.

2. Conductivity (COND). COND is very similar to RI in that all materials provide an inherent response. One major difference is that DR is quite limited for COND, typically in the 10^4 range. In general, a low background is desirable to suppress noise and to enhance the fractional contribution to the response by a given concentration of analyte. Nearly insulating materials like most organic solvents are however not suitable for indirect detection. Since displacement by volume is operative, the 10^4 DR is too small to be useful (in contrast to RI above). Also, since trace amounts of ionic material or even moisture can create large changes in conductivity, it will be hard to stabilize the background when the conductance is low. An appropriate use of COND is in ion chromatography (IC), where the stationary phase is an ion exchanger and an eluting ion is used to move the analyte ion through the column. Indeed, commercial COND detectors for this application function very well.

In the original IC mode,[10] a second column is used to further exchange the ions eluted, so that a low conductivity background is achieved. Since the contribution from the mobile phase (background) is "suppressed", this method represents a direct detection mode even though the signal is derived from an ion generated in the second column, e.g., H^+. It is in effect postcolumn derivatization. In nonsuppressed IC, the second column is not used.[11] Detection is not as good because the eluting ions maintain a large background at the detector at all times. Very good performance is still possible when special eluting ions (those with low inherent conductances) or low concentrations are used. This represents an indirect detection mode because the eluting ion is displaced (charge exchange) by the analyte ions as they elute. The TR is 1 (per equivalent) because of electroneutrality constraints (constant counterion concentration). Even though DR is not as high as in RI, C_m can be made to be low ($\sim 10^{-4}$ M) to provide a respectable LOD. Water, the major component in the mobile phase, really does not contribute at all to the detector response. COND is normally not considered to be an indirect detector even though it does function in that mode in single-column IC. In fact, sensitivity transfer in the indirect mode has been demonstrated with good results.[12]

3. Absorption (ABS). ABS is by far the most popular detector in LC. Normally, it is used as a direct detector to monitor compounds with the right absorption characteristics. We have already mentioned that ABS in the simple volume-displacement mode is useless as an indirect detector. There is a related detection mode called vacancy chromatography.[12] The flowing stream contains all the analytes of interest at their "normal" concentrations. On injection of a sample, the difference in composition between the sample and the flowing mobile phase will be registered as positive or negative peaks at the elution times of the individual components. This however is not a displacement process. The difference in absorbance directly leads to a signal as required by Beer's law. In effect, this mode of detection simply takes advantage of the additive nature of absorbance in a multicomponent mixture. The main point is that, for vacancy chromatography to

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work, the analyte must itself show a response at the detector.

Indirect photometry[13] is a powerful detection scheme in ion chromatography. This is not a volume displacement but a charge displacement. In the nonsuppressed IC mode,[11] one uses a low concentration (~10^{-4} M) of an eluting ion that itself absorbs light. As in the COND detector, the major mobile-phase component—water—does not contribute to the signal at all. Because of local charge neutrality requirements, the analyte ions will displace the absorbing eluting ion on a per equivalent basis and will give rise to a negative peak. Because the eluting ion is already at a low concentration, eq 1 tells us that a useful LOD is possible, typically down to the 10^{-8} M range at the detector.

Indirect photometry also works for other types of LC. Since retention requires partition between the stationary phase and the mobile phase, one can invoke competition between the analyte and a mobile-phase component for indirect detection.[5] Parkin[14] added an UV-absorbing species (e.g., benzamide) in the mobile phase for indirect detection of alkanols and esters. The TR in this case is much less than 1 because the solubility of the mobile-phase component is altered only slightly by the analyte. An analogous situation occurs in ion-pair chromatography,[15] where an UV-absorbing ion is added to the mobile phase. If the added ion has opposite charge to that of the analyte, effectively "on-column" derivatization occurs. If the added ion has the same charge as that of the analyte, then competing equilibria exist between the two and the counterions. The former is similar to IC, with a TR of 1 based on charge neutrality. The latter is similar to solubility modification described above. In general, indirect photometric detection is possible whenever competition of some sort exists between the analyte and the absorbing mobile-phase component. Detection limits are not very impressive, either because the TR is much smaller than 1 or because the concentration of the mobile-phase component cannot be lowered without degrading the separation or the DR.

4. Polarimetry (ORD). Polarimetry is normally designed for highly selective detection of optically active molecules.[16,17] It has also been shown to be a very useful indirect detector.[18,19] The reason is that DR for polarimetry can be very high, in the range of 10^8. This is based on a background rotation of 100° (a fairly typical value for neat liquids of optically active molecules) and a noise level of 10^{-6} °.[20] The crossed-polarizer agreement for the polarimeter is unique in that the noise level is not degraded by the presence of a large background rotation. One simply readjusts the analyzer mechanically to null out the large background. The phototube still sees the same low residual intensity, and the performance is identical with that without the large background.

Because of the large DR, the polarimeter can be used in the volume-displacement mode. The detection of optically inactive compounds by polarimetry is shown in Figure 2. With high-frequency modulation,[20] the LOD is around 1 ng of analyte injected. The limiting factors here are the intensity fluctuations in the light source and temperature fluctuations in the mobile phase.

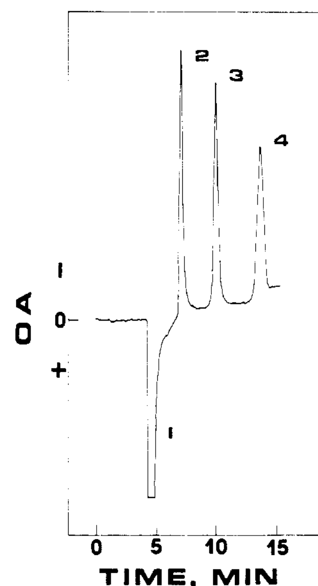

Figure 2. Detection of optically inactive species by polarimetry. Solvent: (+)-limolene; analytes are dioctyl phthalate (2), dibutyl phthalate (3), and diethyl phthalate (4).

In many ways, indirect polarimetry is similar to RI detection. There are however some very important practical differences. Typical RI differences are around 0.1 units, which provide a LOD of 10 ng injected.[9] Typical ORD differences are 100°, since most compounds are not optically active and half of the optically active ones in fact rotate light in the opposite direction. The 1-ng LOD for ORD is thus more the rule rather than the exception. There is also the concern that optically active mobile phases are expensive. They are, but there are several naturally occurring species (e.g., pinene, 3-methylcyclohexanone) that span the range of typical polarities of HPLC solvents. The cost of running these in conjuntion with microbore columns (<1-mm i.d.) is no greater than using UV-grade HPLC solvents in conventional (4.6-mm i.d.) columns. This is because the optically active solvent does not have to be very pure. One simply loses a small amount of sensitivity due to a slightly reduced TR. In fact, there is no reason why one cannot mix optically active mobile phases (to generate the response) with common solvents (to achieve the desired polarity). A 50:50 mixture will only decrease the response by 50%. Finally, it should be noted that indirect polarimetry works well only in the volume-displacement mode. As one decreases C_m (e.g., an optically active eluting ion in ion chromatography), DR also decreases to reduce the detection power.

5. Fluorescence (FL). Fluorescence is a byproduct of absorption. For this reason, indirect FL is very similar to indirect ABS. There are also important differences. First, fluorescence is a more selective process, i.e., not all molecules that absorb light fluoresce significantly, particularly if one fixes an absorption wavelength and an emission wavelength. As discussed above, indirect FL is therefore more broadly applicable.

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Second, while DR for ABS deteriorates as one decreases C_m or if one tries to miniaturize the system (decreased absorption path length), the same need not hold for FL. The same large fluorescence background can readily be maintained in indirect FL by increasing the excitation intensity whenever the concentration or the path length is reduced. We have already seen that lowering C_m is important for good detection. Miniaturization is required to interface with the new generation of separation methods, viz., microcolumn LC[21] and capillary zone electrophoresis[22] (CZE). Those methods are gaining importance with the surge of interest in biotechnology and single-cell studies,[23] where sample sizes are limited. Third, the light intensities reaching the phototube are generally much lower for FL than for ABS. The shot-noise limit is often encountered. For example, one needs at least 10^8 photoelectrons per time constant to approach a DR of 10^4. Fourth, in using high intensity light sources to compensate for low fluorescence intensities, one sacrifices intensity stability and in turn DR. Lasers, for example, are inherently much less stable than conventional light sources. While tungsten lamps can easily produce a DR of 10^4, the laser typically has an intensity stability of only 1 part in 10^2. Electronic compensation by a reference detector is limited to about 10^3 because amplifier gains are never matched exactly. The best DR is obtained by implementing high-frequency modulation.[24] The resulting DR of 10^4 is comparable to that of ABS detectors. Fifth, stray light is a common problem in FL. This is the part of the background that is not "displaced" by the analyte. In effect, the magnitude of TR will be reduced by the same fraction. Sixth, one has to account for the effects of quenching, bleaching, and heating. Quenching by the analyte changes the response, but the net result is enhanced detection because TR is increased. Bleaching decreases the fluorescence intensity and degrades the LOD proportionately. Heating creates thermal fluctuations and reduces the DR.

In the first demonstration of indirect FL detection,[25] LOD was in the 1% (v/v) range for ethanol and methanol and is indicative of a bulk displacement mode. Also reported are LOD in the μg/mL range for certain compounds. The unusual response characteristics there were probably due to a combination of prefilter, primary absorption, self-absorption, and postfilter effects of the analyte[26] and were not a result of indirect FL.

Indirect FL has been applied to ion chromatography, and useful LOD is obtained when the laser intensity is stabilized to provide a DR of 10^4.[24] Sodium salicylate, a strong fluorophore with ion-exchange properties similar to those of common eluting ions in IC, was used as the eluting ion. The results are comparable to those of indirect ABS in IC, since both C_m and DR are similar. It turns out that commercial strong ion exchange columns are not suitable for operation with eluting-ion concentrations below 10^{-4} M because of the high capacity. In principle, weak ion exchangers can be used at low eluent concentrations,[27] although this has not been demonstrated at the 10^{-5} M level. New chromatographic stationary phases are thus needed to take full advantage of indirect FL detection, to allow C_m to be reduced and to improve mass LOD by miniaturization. We note that indirect ABS does not benefit from new stationary phases, since DR decreases with C_m and with decreasing path length.

One approach is based on dynamic modification of standard LC stationary phases. Reversed-phase materials are highly hydrophobic. A quaternary ammonium salt such as cetylpyridinium chloride will attach itself (adsorb) on the stationary phase to provide anion-exchange sites.[28] Since the extent of coverage can be controlled by pH, concentration, and organic modifier, one can generate columns optimized for indirect FL detection. Even silica gel stationary phases can be modified by these salts to become anion exchangers via a double-layer adsorption mechanism.[29] The important point is that once modified, the adsorbed material is not readily washed off by the mobile phase, which is primarily water. With dynamic modification, microcolumns (1-mm i.d.) can be used to provide a LOD of 0.5 ng injected.[29] Using open-tubular capillary columns, detection of 1 pg of NO_3^- has been demonstrated.[28] There is a limit as to how much C_m can be reduced. It turns out that the mobile-phase component can interact with the unmodified parts of the column to compete with the ion-exchange operation. TR therefore becomes less than 1 at low eluting-ion concentrations.

Thin-layer chromatography (TLC) is a widely used analytical technique. It has good separation power because of the possibility of two-dimensional development. Detection, however, has been one of the major problems. Traditionally, tedious and unreliable derivatization procedures are employed because most analytes do not inherently absorb or fluoresce. Indirect FL can be used with TLC to provide universal detection. The same scheme used for anion-exchange LC (above) can be applied to TLC.[30] Even with a chromophore that does not emit strongly in the visible region, and even by using the eye to detect the small decrease in fluorescence on top of a high background intensity (with an estimated DR of less than 10), the order of 5 ng of NO_2^- can be detected. For nonelectrolytes, subnanomolar LOD can be achieved.[31] Examples include alkanols by reversed-phase TLC, bile acids by normal-phase TLC, and digoxin by reversed-phase TLC. TLC is particularly interesting because one can visualize the progress of the separation in real time by indirect FL to optimize the results. Such would have been impossible with other detection schemes for nonabsorbing analytes.

A rapidly developing area in chemical analysis is capillary zone electrophoresis. Although detection by fluorescence (typically with derivatization) and detection by electrochemistry have led to excellent results, there is still a great need for an all-purpose detector. Indirect FL is a good match for CZE. There is no

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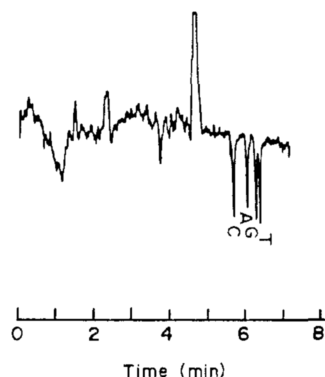

Figure 3. Indirect fluorescence detection of nucleotides (5'-monophosphates) in capillary zone electrophoresis.

problem with the small dimensions of the capillaries. It is nondestructive and is therefore suitable for many preparative applications. Most importantly, there already exists in CZE a buffer that maintains the high electrical-field gradient used for the separation. It is straightforward to use instead a fluorescing ion as the buffer, or as a major component of the buffer. Since all species that are separated by CZE are charged, there is a natural displacement of the charged fluorophore in the buffer by the analytes due to local charge neutrality requirements. This then provides a favorable TR. Electrophoresis is mostly based on mobility of the ions. So even a low C_m (to favor detection) does not significantly alter the separation process.

There are a few practical limitations that require a judicious choice of the electrophoresis conditions for indirect FL detection. As C_m decreases, the buffer capacity of the system also decreases. So, steps like degassing to eliminate dissolved CO_2 are critical. The column resistance also goes up when C_m is lowered. To prevent external electrical breakdown when the resistance of the column is larger than that of the surrounding air, a parallel pass–bank resistor must be used. That also helps in stabilizing the current through the capillary. Electroosmotic flow must be controlled to a reasonable level at low ionic strengths to maintain the high separation efficiency due to electromigration. This can be accomplished by pacifying the walls of the capillary tube, e.g., by silylation. At very low C_m, there can also be additional interactions on the wall involving the fluorophore to affect the displacement process. Finally, one has a limited pH range to work with, since above pH 10 and below 4, OH^- and H^+ respectively will be a significant fraction of the charged species involved in the displacement process.

Indirect FL detection in CZE has been shown for a variety of analytes. Use of 50 μm i.d. tubes, 5-mW excitation at 325 nm (HeCd laser), 2×10^{-4} M salicylate as the buffer, and a DR of only 100 (no intensity stabilization) enables native amino acids to be separated and detected at the 10-fmol level injected.[32] With intensity stabilization to allow a DR of 10^3, nucleotides can be detected at the 50-amol range in 15 μm i.d. tubes.[33] This is shown in Figure 3. Since this is a charge-displacement mechanism, common anions (IO_4^-, HCO_3^-) and even proteins (lysozyme) are also detected at the same low level. It is interesting to note that proteins can in fact be multiply charged at high pH, so that the LOD can be even lower than 50 amol.

Conclusions

Indirect detection allows one to monitor species that normally do not give a response. It has grown from an intellectual curiosity to become an extremely useful tool. For volume displacement, polarimetry gives the best results. For charge displacement, ion chromatography and capillary zone electrophoresis are ideal situations for applying indirect fluorescence detection. LOD surpasses all but the most specialized detection schemes. Future developments in systems specially designed for indirect detection should make these schemes even more powerful.

What are some of the urgent problems that can benefit from indirect FL detection in CZE? Naturally, the 10^{-7} M concentration LOD is not particularly impressive compared with other detection schemes, except of course for its universal response. There are sample limited situations where chemical manipulation before analysis is not desirable. The study of the intracellular fluids of a single cell is a major challenge. The volume fits very well with the column sizes in CZE. In principle, one can obtain a profile of the components of each cell by CZE and explore chemical differences between different cells. Of current interest are protein sequencing and DNA sequencing. In the former, the standard technique requires 1 h per unit because of the complication of gradient LC identification of the amino acid released in each cycle of Edman degradation.[34] Detection is only in the 10-pmol range. In the latter, slab gel electrophoresis is normally used, even though newer atuomated systems have recently been developed. Figure 3 shows that we can identify which of the four bases is involved in a separation that lasts only 1 min. If one can work out the microchemistry related to handling such small DNA or protein samples, indirect FL detection should have a major impact on biology and biochemistry.

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