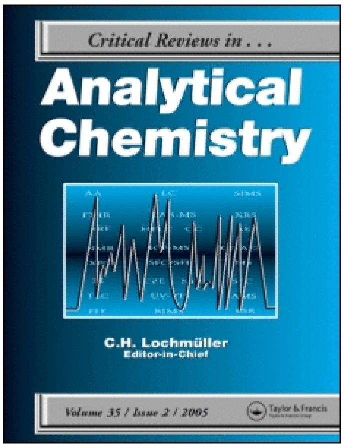

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Indirect Photometric and Fluorometric Detection in High-Performance Liquid Chromatography: A Tutorial Review

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A review of indirect photometric and fluorometric detection in high-performance liquid chromatography (HPLC) is presented. A functional definition of indirect detection, somewhat broader and simpler than what was often used to characterize these techniques, was employed for this review. Emphasis was placed on distinguishing different approaches to indirect photometric and fluorometric detection in HPLC based on the interactions which occur between the analytes and the detectable components either within the column or postcolumn.

Keywords indirect detection, indirect photometric detection, indirect fluorometric detection, high-performance liquid chromatography

INTRODUCTION

High-performance liquid chromatography (HPLC) is a powerful analytical tool when coupled with a suitable detector. Ultraviolet-visible (UV-Vis) absorbance and fluorescence detectors are two of the most popular and convenient HPLC detectors in use today. However, as generally practiced, these approaches to analyte detection require that the analyte possess a functional group which either absorbs UV-Vis light (i.e., has a chromophore) or fluoresces after being exposed to the appropriate wavelength of UV-Vis light (i.e., has a fluorophore). A chromophore or fluorophore may be attached to a non-absorbing or non-fluorescing analyte by reacting with a suitable derivatization reagent. The derivatization reaction is performed either prior to injecting the sample into the chromatographic column (i.e., precolumn) or after the analytes elute from the column but prior to detection (i.e., postcolumn). Many fine examples demonstrating the application of each type of derivatization may be found in the literature (1). A number of issues must be addressed when developing such a derivatization method, including: problems associated with choosing suitable reagents, minimizing time-consuming sample treatments, ensuring complete and/or reproducible product formation, minimizing side-product formation and, in the case of postcolumn derivatization, limiting band broadening due to the presence of a postcolumn reaction volume.

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While direct detection of analytes or suitable derivatives remains the most widely practiced approach to spectroscopic detection in HPLC, a number of other approaches have been developed to detect analytes that lack a chromophore or a fluorophore. These methods, which provide an alternative to direct detection, are often characterized as *indirect* methods of photometric or fluorometric detection.

The general topic of indirect photometric detection was included in the biannual reviews of column liquid chromatography published in *Analytical Chemistry* from 1986 to 1994 (2–6). Several reviews with abundant references on indirect detection, including indirect photometric detection and applications in liquid chromatography, have been published (7–15). Here, the several ways in which indirect detection has been defined, especially as related to indirect photometric and fluorometric detection, are reviewed and a broader definition is introduced. Based on this broader definition, a number of approaches that have been used for indirect photometric and fluorometric detection in HPLC are reviewed and classified according to the types of interactions that occur between the analyte and the detectable component.

DEFINING INDIRECT DETECTION

"Indirect detection" has been defined in many different ways and, in practice, a large number of different approaches have been applied. For example, Small and Miller (16) described the use of indirect photometric detection in ion chromatography. The detection of a UV-Vis-transparent analyte is accomplished by adding light-absorbing ionic species into the mobile phase. The presence of the analyte is monitored by measuring a decrease in the light absorbed by the eluent as the analytes elute from the column. This approach to indirect detection was extended to other chromatographic systems such as reversed-phase chromatography (17–20) and reversed-phase ion-pairing chromatography (21, 22). Jurkiewicz and Dasgupta similarly defined indirect detection as a technique that monitors deficiencies in eluent constituents rather than the emergence of sample components (23). Yeung indicated the key feature of indirect detection methods is that the indirect response measured is due to the absence of a mobile-phase component rather than the presence of the analyte (11). The indirect detection methods highlighted in that review included measuring changes in refractive index, conductivity, light absorption (indirect photometry), polarimetry, and fluorescence (11).

Takeuchi and co-workers (9, 15, 24) defined indirect detection as a method whereby the analytes are visualized by measuring variations in the background signal resulting from the interactions between the analytes and the detectable component or by the detection of a detectable species produced by postcolumn interaction. Postcolumn ion replacement and postcolumn enzyme reactions are included as methods of indirect detection under this definition (9, 15). Verchère and Dona (12) considered indirect detection methods as involving exchange processes where the exchange specifically replaces one species by another without modification of the internal bonds of either. They included some forms of titration and "re-extraction" as examples of indirect detection. The detection of carbohydrates via postcolumn reaction with alkaline cupriammonium to form ternary complexes with enhanced absorbance at 300 nm is also an example of indirect detection under this definition (12).

The many definitions of indirect detection as well as inconvenient cross-referencing among these methods may limit their application. For this reason, a somewhat broader, and simpler, functional definition of indirect detection will be employed for this review. Any method of detection in which the presence of an analyte as it elutes from the column is not measured directly, but instead is inferred by measuring a response due to some other component, will be included here as an indirect method of detection. This brief review will be limited to UV-Vis absorption or fluorescence methods since these are the most popular optical detection methods used in HPLC. Here, indirect photometric and fluorometric detection in HPLC will be distinguished further by whether the interactions between the analytes and the detectable components occur within the column or postcolumn.

INDIRECT DETECTION RESULTING FROM INTERACTIONS OCCURING WITHIN THE COLUMN

Interactions that occur between the analyte and the detectable component, within the column, may change either the concentration of the detectable component in the eluent or the spectral properties of that detectable component. Monitoring changes in the photometric or fluorometric signal of the detectable component as it elutes from the column provides an opportunity to detect the analyte indirectly. Most of the indirect methods of detection covered in the literature describe monitoring changes in the concentration of a detectable component due to the versatility of such an approach. In some cases, however, analyte detection may be also achieved by monitoring changes in the spectral properties of the detectable component (e.g., a shift in the wavelength of maximum absorbance) rather than changes in the concentration of the component.

Indirect Detection Based on Measuring a Change in the Concentration of the Detectable Component in the Eluent

The methods covered in the following three sections are further characterized either as indirect photometric detection or indirect fluorometric detection, depending on whether the property being detected is UV-Vis-absorption or fluorescence. In general, the basis for indirect photometric detection described here is also applicable to indirect fluorometric detection.

Basis for Indirect Photometric Detection. The indirect photometric detection methods described in this section are based on the following principles: (1) The detectable component is continuously introduced into the column with the mobile phase; (2) Equilibrium is established for the detectable component between the mobile and the stationary phases; (3) The equilibrium concentration of the detectable component in the mobile phase provides a constant background level for detection; (4) The presence of the anlayte affects the distribution equilibria within the column that results in a positive or negative change in the detector's response for the detectable component as the analyte elutes. Using this form of indirect detection, injecting a sample results in a peak or peaks being observed for each of the injected analytes as well as one or several additional peaks that are characteristic of the system, called "system peaks." Several studies have been conducted to evaluate the origin of these system peaks (25–37). The detectable component has also been referred to as a "probe" (21, 38–43), "marker" (44, 45), or "visualization agent" (9, 46–48). Most of the detectable components employed to date absorb UV light, though others which absorb in the visible region, such as methylene blue (49, 50), ethyl violet (51), thymol blue (52), methyl red (53), and chlorophyll (a and b) (54) have also been employed. Selection of a suitable detectable component depends primarily upon the analyte and the mode of liquid chromatography providing the separation. A general outline is presented in Table 1.

Ionic analytes have been analyzed by ion-exchange and ion-pairing chromatography with the use of ionic detectable components. In ion-exchange chromatography, the ionic detectable components serve a dual role, affecting the retention of the analytes and providing detection for ionic analytes having the same charge polarity as the ionic detectable components. For anionic analytes, the detectable components used for indirect detection have included: phthalate (16, 44, 45, 55–63), naphthalene sulfonate derivatives (64), 2,4-dihydroxybenzoate (65), p-hydroxybenzoate (66), pyromellitate (67, 68), trimesate (69, 70), nitrate (16, 71–73), iodide (48), hexacyanoferrate (II)

TABLE 1
Outline for the selection of a suitable detectable component based on the analyte and the mode of liquid chromatography

Analyte	Mode of liquid chromatography	Detectable component
Ionic	Ion-exchange	UV-Vis-absorbing ionic species with the same charge polarity as the analyte
Ionic	Ion-pairing	(1) UV-Vis-absorbing hydrophobic ion and non-UV-Vis-absorbing hydrophilic counter-ion
		(2) Non-UV-Vis-absorbing hydrophobic ion and UV-Vis-absorbing hydrophilic counter-ion
		(3) UV-Vis-absorbing hydrophobic ion and hydrophilic counter-ion
Non-ionic	Reversed-phase	Ionic or non-ionic UV-Vis-absorbing compounds

and hexacyanoferrate (III) (74), anthraquinone-disulfonate (75), sulfosalicylate, and 1,2-dihydroxybenzene-3,5-disulfonate (76). Detection for otherwise undetectable analytes that are cations has been achieved with the use of Cu(II) (16, 77), Ce(III) (77–79), aromatic monoamines (55, 80, 81), and 3-hydroxytyramine (82). Anions and cations can also be detected simultaneously with the use of a detectable component which is a cation-anion pair following separation by cation and anion exchange columns arranged in series (16, 83), or a mixed-bed ion-exchange column (84).

Indirect detection has been accomplished in ion-pairing chromatography by adding a detectable ion-pairing (or ion-interaction) reagent to the mobile-phase. The ion-pairing reagent consists of a hydrophobic ion, which enhances the retention of the ionic analyte, and a relatively hydrophilic counter-ion. Three types of detectable, ion-pairing reagents have been reported. These types of reagents are distinguished based on whether the hydrophobic or the hydrophilic ions serve as the detectable species. The first type of ion-pairing reagent consists of a UV-absorbing hydrophobic ion and a non-UV-absorbing hydrophilic counter-ion. UV-absorbing hydrophobic ions include: 1-phenethyl-2-picolinium (21, 22, 85, 86), the naphthalenesulfonates (21, 22, 86–89), protriptyline (86, 89), cetylpyridinium (90), iron(II) 1,10-phenanthroline (91, 92), ruthenium(II) 1,10-phenanthroline (93–95), and ruthenium(II) 2,2′-bipyridine (93). This type of ion pairing reagent provides a means for monitoring the elution of ionic analytes possessing the same charge polarity as the detectable species or the opposite charge polarity of the detectable species. The second type of ion-pairing reagent consists of a non-UV-absorbing hydrophobic ion and a more hydrophilic, UV-absorbing counter-ion. UV-absorbing counter ions which have been employed include: thymolsulphonate (96), 3-nitrophthalate (97), phthalate (98), salicylate (98, 99), sulfanilate (40), 3-hydroxylbenzoate (41), benzoate (100), 1-naphthalenesulfonate (100), naphthalene-1,5-disulfonate (100, 101), methylpyridine (41, 102), papaveraldine (103), and chromate (104). The mechanism for detecting ionic analytes using this type of ion-pairing reagent is similar to that observed for ion-exchange chromatography, where the detectable species provides detection for analytes having the same charge polarity as the detectable species. The third type of ion pairing reagent consists of a hydrophobic ion and a hydrophilic counter-ion, both of which absorb light at the same wavelength, such as: 1-phenethyl-2-picolinium and 3-hydroxybenzoate (38, 86), or 1-phenethyl-2-picolinium and 6-hydronaphthalene-2-sulphonate (86). It has been suggested that the use of this type of ion-pairing reagent provides improved detection sensitivity for compounds that have considerably lower retention than that of the system peak (38, 86).

Separation of non-ionic analytes, also called "non-electrolytes" (14, 105) or "neutral analytes" (21, 41, 43), is often achieved with reversed-phase chromatography. Indirect detection for otherwise undetectable analytes has been achieved by adding components to the mobile phase that absorb either ultraviolet or visible light. Both ionic and non-ionic compounds have been used as mobile phase additives to provide indirect detection of non-ionic analytes. Among the additives that have been used as detectable components are: naphthalene-2-sulfonate (21, 43), 1-phenethyl-2-picolinium (21), 1-methylpyridine (41), nicotinamide (18,105), sulfanilamide (105), isoniazid (105), salicylamide (18, 20), arbutin (hydroquinone-β-D-glucopyranoside) (18), cholecalciferol (20), benzamide (17, 106), n-propyl-p-aminobenzoate (17), theobromine (106), benzene, nitrobenzene, benzonitrile, benzaldehyde, benzyl alcohol (19), methyl blue (49), chlorophyll (a and b) (54), and theophylline (107).

In addition to the specific analytes and the mode of liquid chromatography employed, other factors that may affect the chromatographic separation and the detection sensitivity must be considered when selecting detectable components. These factors include: the molar absorptivity and the concentration of the detectable components in the mobile phase, the pH of the mobile phase, and for ion exchange chromatography, the exchange capacity of the stationary phase. For example, the concentration of the detectable component in the mobile phase and the molar absorptivity at the wavelength of detection determine the background absorbance for indirect photometric detection. Barber and Carr (108) found that for the detector which they employed, the signal-to-noise ratio began to decrease when the background absorbance was above about 1.0 AU (absorbance unit). Above this background absorbance, there was no gain in the signal level for the analytes. Small and Miller (16) suggested the absorbance level of the background should range from 0.2 to 0.8. Generally, a relatively low concentration of the detectable component in the eluent has been employed to avoid a high background absorbance level.

Conversely, there is a lower limit for the concentration of the detectable component in the eluent. A low concentration of the detectable component may result in increased elution time of the analyte and thus increased band broadening (16), assuming the detectable component affects the retention of the analyte. For this reasons, the concentration of the detectable component in the mobile phase and the wavelength of detection must not be considered independently (108). For detectable components that affect the analyte's retention, choosing a different wavelength of detection, where the molar absorptivity of the detectable component is small, a low level of background absorbance for a high concentration of detectable component in the eluent may be achieved, while the desired displacing power of the eluent ion is still maintained. The pH of the mobile phase is another factor that often must be considered. The pH of the mobile phase may affect the degree of ionization and the absorbance properties of the detectable component (109), potentially affecting the displacing power of the eluent ion in ion-exchange chromatography or the level of background absorbance. In ion-pairing and reversed-phase chromatography, the affinity that the detectable component has for the stationary phase, as reflected by its capacity factor, is also critical. Studies have shown that an analyte is detected with maximum sensitivity when its capacity factor is close to that of the detectable component (7, 18, 38, 41, 85, 86, 96). Variables that may affect the capacity factor of the probe, such as the concentration and nature of the organic modifier and the mobile phase buffer, have been studied (90, 110). Studies demonstrating the optimization of the indirect photometric detection system have also been presented in the literature (44, 90, 111, 112).

Application of Indirect Photometric Detection. Over the years several reviews have been published that highlight a variety of applications of indirect photometric detection in HPLC. Crommen (7) reviewed the application of indirect photometric detection in the analysis of amino acids and dipeptides in 1984. Indirect photometric detection of inorganic anions in HPLC was included in a review that was published in 1984 by Haddad (8). Schill and Arvidsson (10) in 1989 reviewed the application of indirect photometric detection methods for biomedical analysis by HPLC. A review with 118 references was published covering the indirect photometric detection of non-electrolytes in HPLC for the 15 years prior to 1998 (14). The application of indirect photometric detection in HPLC for analyzing real samples including foods, body fluids, environmental and pharmaceutical samples was summarized in a review that was published in 1995 by Hayakawa and Yamamoto (13). A summary of some examples of applications of the indirect photometric detection for analyzing real samples using HPLC that were not included in Hayakawa and Yamamoto's review is presented here in Table 2, along with information on the analytes being detected, the mobile phase containing the detectable components, column, linear range, wavelength of detection, and limit of detection/quantitation.

Application of Indirect Fluorometric Detection. If the detectable component, which is added to the mobile phase, fluoresces under the conditions employed, indirect detection can also be accomplished using a fluorescence detector. Su et al. (123) added aniline to the mobile phase to allow the elution of both fluorescent and non-fluorescent polynuclear aromatic hydrocarbons to be detected. Mho and Yeung (124) detected iodate and chloride ions using a mobile phase containing sodium salicylate as the fluorescent agent. They used a double-beam fluorescence detector to increase the stability of the fluorescence signal measured. Sodium salicylate was also used as the detectable component in the mobile phase to provide indirect fluorometric detection for inorganic anions such as acetate and nitrate (125, 126). Non-electrolytes, such as the alkanols, were detected by introducing 2,7-dichlorofluorescein (126) or anthracene (127) into the mobile phase as the detectable fluorescent component.

Gallo and Walters (128) investigated 2-amino-5-methylbenzenesulfonic acid as a detectable, mobile-phase component for indirect fluorometric detection of aliphatic amines such as: butylamine, isobutylamine, and cyclohexylamine. Cerium (III) ion, which has been used for indirect photometric detection by measuring the absorbance level of the eluent at 254 nm (77–79), has also been used for indirect fluorometric detection of sodium, ammonium and potassium ions in urine by exciting at 247 nm and observing the fluorescence at 350 nm (129). One of the primary advantages of using this fluorometric method for indirect detection is that a UV-absorbing matrix will not interfere with monitoring the fluorescence signal if the matrix does not absorb at the excitation or emission wavelength of the detectable component.

Rigas and Pietrzyk (130) added fluorescent ruthenium(II) 1,10-phenanthroline and ruthenium(II) 2,2′-bipyridine complexes to the mobile phase to provide detection of inorganic and organic anions separated with a Hamilton PRP-1 column. Fung and Tam (131) performed an optimization study to improve the detection of inorganic anions when using ruthenium(II) 1,10-phenanthroline as the mobile phase additive. Diode-laser-based indirect fluorometric detection of n-alkyl alcohols was undertaken with the use of a near infrared dye, IR 125, which was added to the mobile phase (132). Indirect fluorometric detection of 11 EPA priority phenols following their separation by reversed-phase capillary HPLC has been reported (133). The detectable component employed in this case was sodium 1-naphthalenesulfonate, which has a maximum fluorescence emission wavelength of 665 nm when excited at 280 nm.

A novel approach to indirect fluorescence detection was described by Jurkiewicz and Dasgupta (23). They added either 4-amino-1-naphthalenesulfonic acid or 6,7-dihydroxy-2-naphthalenesulfonate to the mobile phase in concentrations at which they are strongly self-quenched. The concentration of the detectable components in the mobile phase decreases as the analytes elute from the column, resulting in an increase in the

TABLE 2
Examples of applications of indirect photometric detection based on measuring a change in the concentration of the detectable component in the eluent

Sample	Analyte	Mobile phase	Column	λ^a (nm)	Linear range	LOD/LOQ^b	Ref
Pharmaceutical tablets	Captopril	0.1M potassium hydrogenphthalate (pH 6.0)/methanol/water = 25/150/825 (v/v/v)	Vydac anion-exchange column	280	0.08–1.05 mg/ml	0.70 ng (in 25 μl) (LOQ)	45
Ertapenem sodium bulk drug	Acetate Chloride	1.0 mM p-hydroxybenzoic acid (pH 9.5)/methanol = 99/1 (v/v)	PRP-X100	305	0.0023–0.4663 mg/ml 0.0032–0.6306 mg/ml	0.002 mg/ml 0.003 mg/ml (LOQ)	66
Bulk drug	Alendronate Etidronate Clodronate 2-Phosphono-pyrrolidine Alendronate dimer	1 mM trimesic acid (pH 5.5)	PRP-X100	254	0.1–2 mg/ml	50 ng–250 ng (in 10 μl)	69
Pharmaceutical tablets, solutions, and powder	Citrate	0.875 mM trimesic acid (pH 10.0)	PRP-X100	280	1–12 μg (in 20 μl)	0.26 μg (in 20 μl) (LOQ)	70
Pharmaceutical tablets	Etidronate disodium	7.2 mM nitric acid	IC-Pak HR anion-exchange	240	0.2–0.6 mg/ml	0.001 mg/ml	71
Pharmaceutical tablets	Alendronate sodium Etidronate disodium Clodronate disodium	1.6–12 mM nitric acid	IC-Pak HR anion-exchange	235–245	0.02–0.08 mg/ml 0.08–0.8 mg/ml 0.08–0.8 mg/ml	0.001 mg/ml	72

(Continued on next page)

TABLE 2
Examples of applications of indirect photometric detection based on measuring a change in the concentration of the detectable component in the eluent
(Continued)

Sample	Analyte	Mobile phase	Column	λ^a (nm)	Linear range	LOD/LOQ[b]	Ref
Mouthwash Disinfectant solution	Ethanol Glycerol Isopropanol	67.8 μM sulfanilamide, 0.4 mM nicotinamide, 0.28 mM isoniazid	Hypersil ODS	260	0.15%–0.36% (w/v) 1.57%–2.7% (w/v) 0.1%–0.24% (w/v)	—	105
Injection for veterinary use	Propylene glycol N,N-dimethyl-acetamide	67.8 μM sulfanilamide, 0.4 mM nicotinamide, 0.28 mM isoniazid	Hypersil ODS	260	0.21%–0.36% (w/v) 0.028%–0.048% (w/v)	—	105
Topiramate drug substance	Sulfamate Sulfate	5.8 mM p-hydroxybenzoic acid and 2.5% methanol (pH 9.4)	PRP-X100	310	0.25–6.3 mole % 0.25–18.8 mole %	0.1 mole %	113
Pharmaceutical tablets	Meprobamate	1 mM benzoic acid in a mixture of 0.05M phosphoric acid (pH 1.9) and methanol (70:30, v/v)	Lichrospher RP-18	273	0.5–8 mg/ml	—	115
		0.49 μM cinnamic acid in a mixture of phosphate buffer (pH 4.8) and methanol (60:40, v/v)		273	0.25–4 mg/ml	—	115
Foods	Cyclamate	10–30 μM Methyl Red in 0.02 M phosphate buffer (pH 7.0)/ methanol =3/2 (v/v)	Inertsil ODS-3	433	—	0.14 mM	53
Foods	Free, bound and total sulphites	0.15g/L potassium hydrogen-phthalate (pH 5.7)	Supelcosil LC-SAX	280	0–300 ng (in 20 μl)	0.42 ng (in 20 μl)	61
Blood	Bromide	0.01M citrate buffer-acetonitrile (80:20, v/v) containing 0.1 mM papaveraldine perchlorate and 2.6–4.5 mM tetrabutylammonium hydroxid	μBondapak C18	325	1–25 nmol (in 20 μl)	3.2 mg/l	103
Serum samples Tap water	Chloride Bromide Nitrate	1 mM sodium iodide and 0.3 mM tartaric acid (pH 3.3)	Octadecylsilica immobilized with bovine serum albumin	225	0.1–1.0 mM	3.3 μM 3.2 μM 3.9 μM	48

Drinking water	Chloride Nitrate Sulphate	2 mM 3-nitrophthalic acid (pH 4.0) containing 18% (v/v) acetonitrile	Hypersil-5-ODS coated with cetyl-trimethylammonium bromide	325	20–2500 ppm	—	97
Tap water	Chloride Nitrate Sulphate	1 mM sodium salicylate (pH 5.8) containing 5% acetonitrile	Develosil ODS-3K coated with cetyl-trimethylammonium bromide	230	0.02–1 mM	0.02 mM	99
Sediment samples	Sulfate	1.6 mM sulfobenzoic acid and 0.2 mM trimesic acid (pH 5.5)	Nucleosil SB anion exchange	258	1–100 nmol (in 100 μl)	600 pmol (in 100 μl)	109
Tap water Drinking fountain Pond water Lowland river Upland stream Underground stream Drainage from China clay works	Nitrate	1 mM potassium hydrogenphthalate (pH 3.95)	Partisil 10 SAX	265	3.67–29.2 μg/ml	0.1 μg/ml	116
Pore water	Sulfate	0.4 mM 5-sulfoisophthalic acid (pH 4.5)	Nucleosil SB anion exchange	240	0.3–25 ppm	20–50 pmol (in 20–200 μl)	117
Atmospheric waters	Sulfite Sulfate Hydroxymethane sulfonate Chloride Nitrate Nitrite	0.5 mM potassium hydrogen-phthalate-0.015 %triethanolamine-3% methanol (pH 7.9)	Eclipse XDR-C18 coated with cetylpyridinium chloride	265	19–430 μM 6.7–430 μM 3.8–430 μM 1.9–430 μM 9.7–430 μM 4.8–430 μM	19 μM 6.7 μM 3.8 μM 1.9 μM 9.7 μM 4.8 μM	63
Lysimeter leaching test samples	Acetic Lactic Propionic Pyruvic	0.5 mM salicylic acid pH 5.5 containg 5% (v/v) acetonitrile	Supelcosil LC-18 coated with cetylpyridinium chloride	293	1–3 μg/ml	0.06 μg/ml 0.07 μg/ml 0.09 μg/ml 0.10 μg/ml	118

(Continued on next page)

TABLE 2
Examples of applications of indirect photometric detection based on measuring a change in the concentration of the detectable component in the eluent
(Continued)

Sample	Analyte	Mobile phase	Column	λ^a (nm)	Linear range	LOD/LOQ[b]	Ref
Gold process effluents	Chloride	0.5 mM 1,3,5-benzene-tricarboxylic acid (pH 7) containing 2.5% (v/v) n-butanol	Supelcosil LC-18 coated with cetylpyridinium chloride	254	2–20 μg/ml	0.02 μg/ml	118
	Cyanate					0.05 μg/ml	119
	Sulphate					0.05 μg/ml	
	Thiosulphate					0.07 μg/ml	
	Thiocyanate					0.1 μg/ml	
Liquid and solid chemical wastes	Fluoride	2 mM potassium hydrogenphthalate (pH 5.0)	PRP-X100	272	0.03–10 μg/ml	0.03 μg/ml	120
	Phosphate				0.06–16 μg/ml	0.06 μg/ml	121
	Chloride				0.05–15 μg/ml	0.05 μg/ml	
	Bromide				0.1–30 μg/ml	0.1 μg/ml	
	Iodide				0.7–100 μg/ml	0.7 μg/ml	
	Sulfate				0.07–50 μg/ml	0.07 μg/ml	
Residues of low explosives	Nitrite	4 mM benzyltributylammonium chloride/5 mM phosphate buffer (pH 4.6)/0.25 mM hexane-sulfonate	Lichrospher RP-18	222	0.05–10 mM	2.8 ppm	122
	Nitrate					0.8 ppm	
	Chlorate					21.3 ppm	
	Sulfate					28.4 ppm	
				262		48.3 ppm	
						8.5 ppm	
						10.7 ppm	
						14.2 ppm	

[a] λ: wavelength of detection.
[b] LOD: limit of detection; LOQ: limit of quantitation. (The values listed are LOD unless otherwise specified).

fluorescence signal. By monitoring this signal, indirect fluorometric detection of fluoride, chloride, bromide, iodine, thiocyanate, and sulfate was achieved. This approach to indirect detection may be useful in applications where it is possible to operate at a relatively low level of background fluorescence.

Indirect Detection Based on Measuring a Change in a Spectroscopic Property of the Detectable Component in the Eluent

Another approach to indirect detection is based on monitoring a change in a spectroscopic property of the detectable component in the eluent. The spectral change results from an interaction between the detectable component and the non-detectable analyte in the eluent. For example, the absorbance of phenolphthalein decreases when it forms an inclusion complex with cyclodextrin (134). Indirect photometric detection of several cyclodextrins was achieved by adding phenolphthalein to the mobile phase as the detectable component (135, 136). However, it was proposed that the mechanism of detection was more complex than this since cyclodextrins disturb the partitioning of phenolphthalein in the column, which also contributes to indirect detection by producing a change in the concentration of the detectable component in the mobile phase (135). Since the absorbance of iodine and the fluorescence intensity of 2-p-toluidinyl-6-naphthalenesulfonate are also enhanced by the presence of cyclodextrins, iodine (137) and 2-p-toluidinyl-6-naphthalenesulfonate (138, 139) have also been added to the mobile phase as detectable components for indirect photometric and fluorometric detection of the cyclodextrins, respectively. The inclusion complexes formed between the cyclodextrins and the detectable component are due to hydrophobic interactions, hydrogen bonding and/or non-specific van der Waals forces (137), distinguishing this detection approach from chemical derivatization, which involves the formation of chemical bond(s) between the analyte and the derivatization reagent. For this reason the methods discussed in this section are considered to be forms of indirect detection. Indirect detection of cyclodextrins can also be achieved by introducing the detectable components postcolumn, a topic to be discussed in a later section.

INDIRECT DETECTION RESULTING FROM INTERACTIONS OCCURING POSTCOLUMN

This approach to indirect detection is easily confused with direct detection following postcolumn derivatization. Direct detection of analytes in HPLC may be achieved by postcolumn chemical derivatization of the analyte. Postcolumn chemical derivatization is accomplished by introducing a reagent, containing the detectable feature, which reacts with the analyte as it elutes from the column. The key distinguishing feature of postcolumn chemical derivatization is that the added reagent undergoes a chemical reaction with the analyte to produce a third compound that is detectable. The derivatization reaction is given by the equation:

$$A + B \rightarrow C$$

where A is the analyte, B is the derivatization reagent, and C is the product, which may also be called "derivatized A."

By contrast, indirect detection resulting from postcolumn interactions does not involve detecting the analyte or a derivatized version of the analyte directly. Instead the presence of the analyte is inferred by monitoring the presence or absence of a detectable species in the eluent. The presence or absence of the detectable species occurs after the postcolumn interactions take place between the analyte and a component introduced either pre- or postcolumn. Thus, postcolumn interaction does not indicate the component that interacts with the analyte must be introduced postcolumn, but instead that the interaction occurs postcolumn. In the following sections some examples will be given where the interactions between the analyte and a component, which is introduced precolumn, actually occur postcolumn. Such interactions may be induced by a catalyst, such as enzyme, which is presented postcolumn. In this review, post-column interactions are classified based on the different approaches that are used to produce or eliminate the species which is ultimately monitored.

Interaction Mode: $A + ML \rightarrow MA + L$

Here, A is the analyte and ML is a metal-ligand complex where M represents the metal ion and L represents the ligand. The coordination number and net charge of the metal-ligand complex are different for different types of metal ions and ligands, though this will be ignored here and in the following sections to simplify the discussion. The metal-ligand complex is introduced postcolumn and will be called a "postcolumn interaction component" for convenience. Detection of the analyte is provided by monitoring either the absence of ML or the presence of L, either of which may provide a detectable signal indicating the presence of the analyte. The analyte, A, and product, MA, are not detectable under the experimental conditions. Depending on the spectral properties of ML and L, indirect detection of the analyte may be achieved by measuring either a decrease or an increase in the absorbance or fluorescence intensity, as shown in Figure 1. A summary of applications based on this approach to indirect detection is presented in Table 3. In the following sections, these applications are described in more detail.

Measuring a Decrease in Absorbance or Fluorescence Intensity. When utilizing this approach to indirect detection, the analyte is detected indirectly by monitoring a decrease in the absorbance or fluorescence intensity of the solution. For example, if the metal-ligand complex, ML, absorbs or fluoresces more strongly at a given wavelength than the ligand, L, then the presence of analyte A is indicated by a decrease in the absorbance or fluorescence intensity due to ML.

Yoza et al. (140) employed this approach when they used the colored, copper complex of methylthymol blue to detect the elution of aminopolycarboxylic acids. A solution containing

TABLE 3
Examples of applications based on the indirect detection approach: A + ML → MA + L

Analyte (A)	ML	L	Signal monitored	Linear range	LOD[f]	Ref
Aminopolycarboxylic acids	Copper-methymol blue	Methylmol blue	Decrease in absorbance of ML	—	0.1 mM	(140)
Clodronate and its esters	Thorium-EDTA-xylenol orang	Xylenol orange	Decrease in absorbance of ML	0.3–16 mg/l	0.3–1.4 mg/l	(141)
Galactitol	Molybdate-chloranilate	Chloranilate	Decrease in absorbance of ML	0–100 mg/l	10.5 mg/l	(142)
D-Mannitol					16 mg/l	
D-Glucitol					17.5 mg/l	
D-Arabinitol					24 mg/l	
Xylitol					36 mg/l	
Galactitol	Molybdate-chloranilate	Chloranilate	Increase in absorbance of L	0–100 mg/l	17 mg/l	(142)
D-Mannitol					26 mg/l	
D-Glucitol					28 mg/l	
D-Arabinitol					38 mg/l	
Xylitol					59 mg/l	
Phosphorus oxo acids, fluoride, sulfate, bisphosphonates, N-(phosphonomethyl)-glycine, aminomethylphosphonic acid	Aluminum-morin	Morin	Decrease in fluorescence of ML	39–824 ng	4–15 ng	(143) (145)
Cysteine	8-Hydroxyquinoline-5-sulfonic-Cd(II)	8-Hydroxy-quinoline-5-sulfonic acid	Decrease in fluorescence of ML	0–430 μM	0.1 μM	(146)
Homocysteine				0–370 μM	0.1 μM	
Glutathione				0–430 μM	0.2 μM	
Cystine				0–100 μM	0.6 μM	(147)
Homocystine				0–150 μM	0.3 μM	
Glutathione disulfide				0–200 μM	0.3 μM	
Biotin Biocytin	Avidin-2,6-ANS[a]	2,6-ANS[a]	Decrease in fluorescence of ML	1–200 μM	0.5 μM	(148)
Biotin	Avidin-HABA[b]	HABA[b]	Increase in absorbance of L	0.01–2 mM	7.3 μM	(149)
Biocytin					9.7 μM	
Organosulphur compounds	Palladium(II)-calcein	Calcein	Increase in fluorescence of L	Two- to three-order concentration range	0.5–1.0 ng (in 100 μl)	(150)
D-Myo-1,2,6-inositol trisphosphate	Fe(III)-methylcalcein blue	Methylcalcein blue	Increase in fluorescence of L	10–1000 ng 3–100 ng (in 10 μl)	10 ng 3 ng (in 10 μl)	(151)
Diamines, polyamines						
Amino acids						
Aminoglycoside antibiotics						

(*Continued on next page*)

TABLE 3
Examples of applications based on the indirect detection approach: A + ML → MA + L *(Continued)*

Analyte (A)	ML	L	Signal monitored	Linear range	LOD[f]	Ref
Amino sugars	Copper-tryptophan	Trytophan	Increase in fluorescence of L	5–1000 pmol 25–1000 pmol 29–586 ng 0.5–10 nmol (in 10 μl)	5–10 pmol 3.8–5.4 pmol 4.2–14.5 ng 0.15–0.30 nmol (in 10 μl)	(152) (155)
Amino sugars	Copper-5-MTP[c]	5-MTP[c]	Increase in fluorescence of L	0.5–10 nmol (in 10 μl)	0.15–0.30 nmol (in 10 μl)	(155)
N-(phosphonomethyl)-glycine Aminomethlphosphonic acid	Copper-APA[d] Copper-ADT[e]	APA[d] and ADT[e]	Increase in fluorescence of L	2 ng–20 μg 4–400 μg (in 200 μl)	8.7 μg/l 6.1 μg/l	(156)

[a]2,6-ANS: 2-anilinonaphthalene-6-sulfonic acid.
[b]HABA: 2-[4′-hydroxyphenylazo]benzoic acid.
[c]5-MTP: DL-5-methoxytryptophan.
[d]APA: N-(9-anthrylmethyl)-2-pyridylmethylamine.
[e]ADT: N-(9-anthrylmethyl)2-diethylenetriamine.
[f]LOD: limit of detection.

copper methylthymol blue (i.e., the ML component) was introduced postcolumn. The molar absorptivity of this complex differs from that of the free ligand at 595 nm. The presence of the aminopolycarboxylic acids in the eluent was detected by monitoring the decrease in absorbance at 595 nm. Another example of this mode of indirect detection is monitoring the elution of the compound clodronate and some of its esters using a mixed-ligand complex, thorium-EDTA-xylenol orange, as the postcolumn interaction component (141). Clodronate and its esters, which were separated by anion-exchange chromatography, interact with this mixed-ligand complex to release the ligand, xylenol orange, resulting in a decrease in the absorbance at 550 nm.

Dona and Verchère (142) detected the presence of alditols utilizing a molybdate-chloranilate complex as the postcolumn interaction component. The molybdate-chloranilate complex has a maximum absorbance at 347 nm while the free chloranilate ligand has a maximum absorbance at 318 nm. The presence of the alditols in the eluent is indicated by a decrease in the level of absorbance at 347 nm. It should be noted that the presence of the analyte can also be detected by monitoring an increase in absorbance at 318 nm, due to an increase in the concentration of the free chloranilate ligand in solution (additional examples of this mode of indirect detection will be covered in the next section). In this case, the limit of detection based on measuring the decrease in absorbance at 347 nm was found to be better than when measuring the increase in absorbance at 318 nm. Pietrzyk and coworkers (143–145) used an aluminum-morin complex as the postcolumn interaction component to detect the presence of phosphorus oxo acids, fluoride, sulfate, bisphosphonates, and N-(phosphonomethyl)glycine and its metabolite, aminomethylphosphonic acid. The aluminum-morin complex fluoresces more intensely at 505 nm, when excited at 420 nm, than does the free ligand, morin. Complexation of the analytes by the aluminum ion results in a decrease in the concentration of the aluminum-morin complex in the eluent. The resulting decrease in the fluorescence intensity of the solution in the detector flow cell indicates the presence of the analytes in the eluent. Pelletier and Lucy (146) monitored the elution of thiols by indirect fluorometric detection. They mixed a solution containing the fluorescent complex, 8-hydroxyquinoline-5-sulfonic-Cd(II), with the eluent from the column. The eluting thiols form a complex with the Cd(II) ion, resulting in a decrease in the fluorescence intensity at 510 nm. This approach was also employed for the detection of disulfides by first reducing the disulfides to the corresponding thiols following elution from the column but prior to mixing with a solution containing the 8-hydroxyquinoline-5-sulfonic-Cd(II) complex (147). Reduction of the disulfides was accomplished by mixing the eluent with tris(2-carboxyethyl)-phosphine.

An example of a complex that is not a metal-ligand complex but which has been used in a similar manner for indirect fluorometric detection is avidin-2,6-ANS (2-anilinonaphthalene-6-sulfonic acid) (148). This complex has been utilized as the postcolumn interaction component for the indirect detection of biotin and biocytin. Biotin and biocytin form complexes with avidin, thereby releasing 2,6-ANS from the fluorescent complex. The resulting decrease in the fluorescence intensity of the solution at 438 nm indicates the presence of these analytes in the eluent.

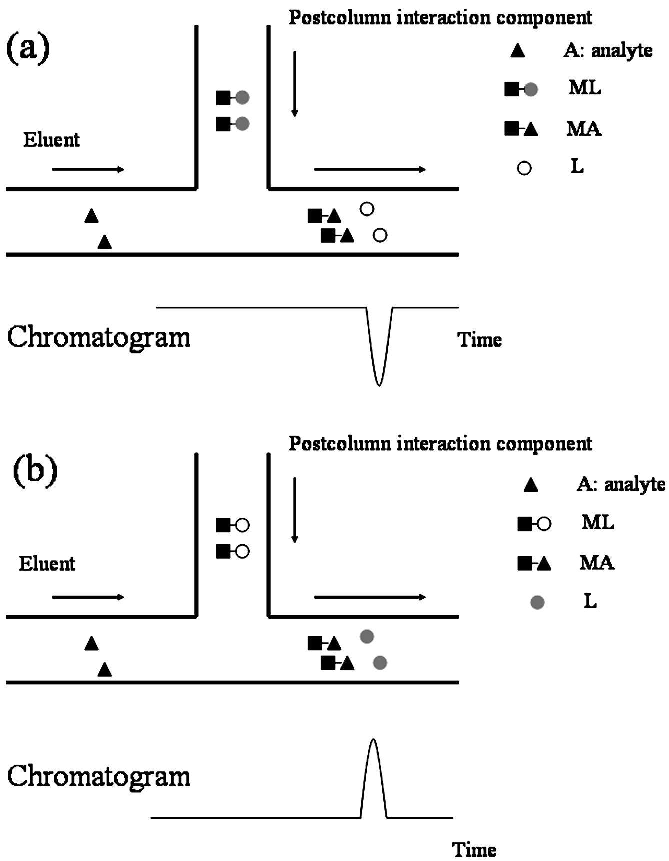

FIG. 1. Indirect detection resulting from interaction: A + M → MA + L: (a) Measuring a decrease in absorbance or fluorescene intensity. (b) Measuring an increase in absorbance or fluorescence intensity. Solid circle for L indicates the analyte (L) either absorbs UV-Vis light or fluoresces. Open cicle for L indicates the analyte (L) neither absorbs UV-Vis ligt nor fluoresces.

Measuring an Increase in Absorbance or Fluorescence Intensity. In this section examples are described where the presence of the analyte is inferred by observing an increase in the measured signal. Here the ligand L absorbs more strongly or fluoresces more intensely at a given wavelength than the postcolumn interaction component, ML. Indirect detection of the analyte, A, under such circumstance can be achieved by measuring the increase in either the absorbance or fluorescence intensity due to the presence of component L.

This approach was employed by Przyjazny et al. (149) to detect the elution of biotin and biocytin from an HPLC column. The detection of analytes was achieved by monitoring the increase in absorbance at 345 nm due to the presence of 2-[4′-hydroxyphenylazo]benzoic acid (HABA) in the eluent, which was released from an avidin-HABA complex introduced postcolumn. The molar absorptivity at 345 nm is higher for the free HABA in solution than for the avidin-HABA complex. HABA is released from the avidin-HABA complex as it interacts with biotin or biocytin as they elute from the column. Thus, the increase in absorbance at 345 nm indicates the presence of either biotin or biocytin.

Werkhoven-Goewie et al. (150) mixed a solution containing a palladium (II)-calcein complex with the eluent to analyze urine and serum samples containing organosulphur compounds. The fluorescence of calcein is quenched efficiently by palladium(II) ion. In the presence of the organosulphur compounds, which compete for the palladium(II) ion, calcein is released from the palladium(II)-calcein complex, resulting in an increase in the fluorescence intensity of the eluent. Similarly, a weakly fluorescent complex of Fe(III) ion with methylcalcein blue was the postcolumn interaction component used for indirect detection of D-myo-1,2,6-inositol trisphosphate (151). In this example, the analytes form stronger complexes with Fe(III), which releases highly fluorescent methylcalcein blue into solution.

Yang and Tomellini (152) introduced a copper(II)-tryptophan complex postcolumn to determine aliphatic biogenic polyamines and diamines. The fluorescence of tryptophan is quenched when complexed with copper(II) ion. These analytes bind with copper to release the fluorescent tryptophan from the copper(II)-tryptophan complex providing the fluorescence response. This approach to indirect detection was extended to the analysis of amino acids (153) and formulated aminoglycoside antibiotics (154).

Shen et al. (155) evaluated 5-hydroxy-L-trytophan and DL-5-methoxytryptophan and found that DL-5-methoxytryptophan is a suitable alternative to tryptophan for this approach to indirect detection. Indirect detection for three mono-amino sugars: glucosamine, galactosamine, and mannosamine was demonstrated based on this approach (155). Tanaka et al. (156) employed a copper(II) complex of either N-(9-anthrylmethyl)-2-pyridylmethylamine (APA) or N-(9-anthrylmethyl)-diethylenetriamine (ADT) as the postcolumn interaction components for the detection of N-(phosphonomethyl) glycine and aminomethylphosphonic acid. The fluorescence of APA or ADT is quenched when complexed with copper(II). The fluorescence of APA or ADT recovers indicating the presence of the analytes after the analytes in the eluent mix with these postcolumn interaction components.

Another variation of this general approach to indirect detection, called postsuppressor ion replacement (157–159), employs equipment and columns similar to what is used in suppressed-ion chromatography. The primary difference between this approach and the ones described in the previous paragraph is that another column is used to introduce ML to replace eluting solute ions or co-ions, A, with a "replacement ion," L. Here M is not a metal ion but a co-ion of L. The concentration of the replacement ion in the eluent is monitored by measuring the UV-Vis absorbance or fluorescence. Dasgupta included this approach as a postcolumn technique for detection in ion chromatography (160). Common replacement ions include Ce(III), anthranilate (157), β-naphthalenesulphonate (158), iodate and nitrate (159). Using this approach, anions such as chloride, bromide, sulfate, phosphate, formate, acetate, and citrate (157, 159), and cations such as lithium, potassium, sodium, and ammonium (158, 159) have been detected in the eluent.

Interaction Mode: $A + ML \rightarrow A' + M'L$

Indirect detection based on a redox reaction occuring between the analyte, A, and the metal ion, M, of a complex, ML, which is mixed with the eluent has been demonstrated. Due to differences in the oxidation states of the metal ion, the reactant, ML, and the product, M'L, have different maximum absorption wavelengths or fluorescence emission wavelengths. Indirect detection of the analyte is accomplished by monitoring either an increase or decrease in absorbance or fluorescence due to the presence of M'L.

The use of a Cu(II)-2,2′-bicinchononate complex to detect sugars and uronic acids (161–166) is an example of this mode of indirect detection. The analytes reduce copper(II) ion in the complex to copper(I) ion. Copper(I) ion complexes with 2,2′-bicinchononate to form Cu(I)-2,2′-bicinochoninate, which has a maximum absorption at 562 nm. Thus, the reducing analytes are detected by monitoring the increase in absorbance at 562 nm. In another example, a peptide, dynorphin A, reduces tris(2,2′-bipyridyl)ruthenium(III) to tris(2,2′-bipyridyl)ruthenium(II). The tris(2,2′-bipyridyl)ruthenium(II) complex fluoresces when excited by visible light indicating the presence of dynorphin A in the eluent (167).

Similarly the presence of analytes that are capable of oxidizing the component which is added to the eluent can also be detected. For example, Mullertz et al. (168) detected phospholipid hydroperoxides by mixing a colorless Fe(II)-thiocyanate solution with the eluent. The hydroperoxides oxidize the Fe(II) to Fe(III) resulting in a violet-colored Fe(III)-thiocyanate complex which absorbs at 505 nm. An increase in the absorbance at this wavelength indicates the presence of the complex, and indirectly, the presence of the phospholipid hydroperoxides in the eluent.

Interaction Mode: $A + B \rightarrow A' + B'$

The methods of indirect detection described here are similar to those employing a metal ion in the redox reaction except the postcolumn interaction component, B, for these methods is not a metal-ligand complex.

Katz et al. (169–171) developed a method for monitoring the elution of reducing agents, including organic acids and carbohydrates, based on the production of fluorescent Ce(III) ion in aqueous solution. The analytes undergo a redox reaction with Ce(IV) ion as they elute from the column, to produce the fluorescent Ce(III) ion. Detection of these analytes is achieved by monitoring the fluorescence of Ce(III) produced at 350 nm when excited at 260 nm. Another approach is based on producing the UV-Vis absorbing or fluorescent compound to be detected via a redox reaction, which is catalyzed by enzymes immobilized postcolumn (172–179).

The enzymes are covalently bound to aminopropyl-controlled pore glass (CPG) (172) or tresylate-poly(vinyl alcohol) beads (178), which are packed into PTFE tubing (172), or a stainless-steel column (178). For example, the fluorescence of reduced nicotinamide-adenine dinucleotide(NADH) at 470 nm, when excited at 365 nm, is commonly monitored to indicate the presence of the reducing analytes in the eluent. NADH is produced when the redox reaction between the reducing analytes and nicotinamide-adenine dinucleotide (NAD) is catalyzed by an immobilized enzyme.

Bile acids have been analyzed by micro-HPLC coupled with a fluorescence detector using this approach to indirect detection (172, 173).

The presence of the bile acids in the eluent is indicated by the fluorescence signal resulting from reduced nicotinamide-adenine dinucleotide(NADH), which is produced by the redox reaction between NAD and the bile acids catalyzed by hydroxysteroid dehydrogenase. The same approach has been applied to the detection of hydroxysteroids (174), glycerol (175), and branched-chain amino acids (176–178) with the use of appropriate enzymes. NADH instead of NAD has been employed for detecting oxidizing agents such as sucrose and fructose (179). To provide a detectable signal for these sugars, an enzyme, sorbitol dehydrogenase, was used to catalyze the reduction of the ketones of sucrose and fructose and the oxidization of the fluorescent NADH to produce the non-fluorescent NAD. The decreased fluorescence intensity of the solution indicates the presence of these analytes as they elute from the column. An interesting feature of this approach to indirect detection is that although the redox reaction takes place postcolumn, NAD or NADH may be added to either the mobile phase (172, 173, 175, 178, 179) or the eluent (174, 176, 177).

One advantage of pre-mixing the NAD or NADH with the mobile phase is the elimination of noise due to pulsation introduced by the pump adding NAD or NADH postcolumn, thereby providing increased sensitivity (172). However, adding NAD or NADH into the mobile phase may affect the chromatographic separation. Introducing the postcolumn interaction component in the mobile phase may not be practical if the chromatographic conditions and the conditions required for the postcolumn redox reaction are so different. The same approach for indirect detection has also been used to detect hydrophilic organic peroxides (180). In this case, an enzyme, horseradish peroxidase, immobilized postcolumn, was used to catalyze the oxidation of (p-hydroxyphenyl)acetic acid by the peroxides to produce a fluorescent biphenyl derivative.

A more complicated strategy for indirect detection, requiring the use of two immobilized enzymes, has been employed to produce a UV-Vis-absorbing or fluorescent species. The purpose of the first enzyme is to oxidize the analytes to produce hydrogen peroxide. The second enzyme catalyzes the oxidation of the postcolumn interaction component by the hydrogen peroxide to form a UV-Vis-absorbing or fluorescent species.

An example demonstrating this approach is the determination of hypoxanthine and xanthine by reversed-phase HPLC coupled with an immobilized enzyme reactor and a fluorescence detector (181, 182). The postcolumn interaction component is p-hydroxyphenylacetic acid. Hypoxanthine and xanthine are oxidized in the presence of immobilized xanthine oxidase as they elute from the column to produce hydrogen peroxide. The resulting hydrogen peroxide reacts with p-hydroxyphenylacetic acid in the presence of the immobilized peroxidase to produce the highly fluorescent product 6,6′-dihydroxy-3,3′-biphenyldiacetate. The postcolumn interaction component, p-hydroxyphenylacetic acid, is mixed with the eluent solution after the hydrogen peroxide is produced. This method was also employed to detect sugars such as stachyose, raffinose, melitiose, and galactose (183). The same approach has been employed to analyze urine samples containing 1,5-anhydro-D-glucitol (184). The postcolumn interaction component used was the sodium salt of N-(carboxymethylaminocarbonyl)-4,4′-bis(dimethylamino)-diphenylamine (also called DA-64). In this case, the hydrogen peroxide produced by the oxidization of the 1,5-anhydro-D-glucitol reacts with DA-64 to produce Bindshedler's Green, which has a maximum absorbance at 727 nm.

Interaction Mode: $A + B \rightarrow A\text{–}B^*$

This approach to indirect detection is based on measuring a decrease or increase in the absorbance or fluorescence intensity of the postcolumn interaction component, B, due to the presence of analyte A. Here, B^* means a change of a spectroscopic property of B resulting from an interaction between A and B. Earlier, examples were presented describing the detection of several cyclodextrins due to the enhanced absorbance of UV-Vis-absorbing compounds or the fluorescence intensity of fluorescent compounds in the presence of cyclodextrins (135–139). In those cases, the detectable components were introduced in the mobile phase. For this approach to indirect detection of cyclodextrins, the detectable components are introduced postcolumn. Phenolphthalein and 2-p-toluidinyl-6-naphthalenesulfonate are examples of detectable components which have been introduced postcolumn for detecting the elution of cyclodextrins (138, 185).

Proteins have also been detected indirectly by postcolumn introduction of the fluorescent compounds, either 2-p-toluidinyl-6-naphthalene sulfonate or 1-anilino-8-naphthalene sulfonate, after separation by size-exclusion chromatography (186). Indirect detection for these proteins is based on monitoring an increase in the fluorescence intensity of the detectable component resulting from hydrophobic interactions with the proteins. Stalikas et al. (187) developed a method for monitoring the elution of nitrite and nitrate after ion chromatographic separation. A decrease in the fluorescence intensity of tryptophan, the postcolumn interaction component, due to dynamic queching of the fluorescence of tryptophan by nitrite and nitrate ions in the eluent, is indicative of the presence of these ions.

CONCLUSIONS

As demonstrated by the many examples presented in this review, indirect photometric and fluorometric HPLC detection provides a reasonable alternative to direct detection for analytes that do not possess native chromophores or fluorophores. Many different types of interactions between the analyte and the detectable component have been exploited to provide different approaches to indirect detection for a wide range of analytes. While general approaches to indirect detection have been presented, a detailed review of the literature shows the choice of detectable component, mode of interaction, and specific experimental conditions will often need to be developed and adapted for specific applications.

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