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REVIEW

APPLICATION OF INDIRECT DETECTION METHODS IN BIOMEDICAL ANALYSIS

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1. INTRODUCTION

Liquid chromatography is today the dominant separation technique in biomedical analysis since it leads to highly efficient isolations and high detec-

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tion sensitivity However, the sensitivity is dependent on the properties of the analyte It must contain structural elements that give rise to high absorbance, fluorescence or electrochemical activity since the most sensitive detection techniques then can be applied If no such structures are present, the detection problem can be solved by pre- or post-column derivatization. This adds a time-consuming step or a technical complication to the analytical procedure A third, technically much simpler possibility is the so-called indirect detection method, which is based on the use of a mobile phase with detector response in the chromatographic system

2 BASIC PRINCIPLES OF INDIRECT DETECTION

Indirect detection methods have long been used for ionic compounds in liquid–liquid systems with an aqueous stationary phase and a mobile phase of low polarity. An ion with detectable properties is added to the stationary phase, and an analyte with the opposite charge will then be distributed to the mobile phase as an ion pair, which is detectable owing to the properties of the counter-ion The design and properties of such systems have been presented by, among others, Crommen et al [1], Crommen [2], Hackzell and Schill [3] and Hackzell et al [4] An example is given in Fig 1 [1]

Reversed-phase chromatography is, however, the main field of application of the indirect detection technique, and in such system it can be applied to charged as well as uncharged analytes The basic principle is very simple A

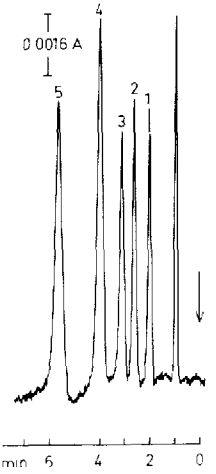

Fig 1 Separation of dipeptides Mobile phase, chloroform–1-pentanol (19 1), stationary phase, 0 1 *M* naphthalene-2-sulphonate (pH 2 3), support, LiChrospher SI-100, detection, 254 nm Peaks 1 = leucylleucine, 2 = phenylalanylvaline, 3 = valylphenylalanine, 4 = leucylvaline, 5 = methionylvaline

compound with detectable properties and affinity for the solid phase is included in the system. Analytes injected into the system will then give rise to detector response owing to their influence on the distribution of the detectable system component (the probe).

The response pattern in the liquid–solid systems depends on the properties of the solute and the probe, and it deviates significantly from that given by analytes with inherent detectable properties. UV absorption is one of the most frequently used detection principles, and it has been applied in the example given in Fig. 2 [5] The mobile phase contains a quaternary ammonium ion, 1-phenethyl-2-picolinium, as detectable component (probe) The analytes are carboxylic acids, mainly present in anionic form Two kinds of peaks appear. one peak for each of the analytes and one additional peak that is characteristic for the chromatographic system (a system peak) Furthermore, the solute peaks are negative when eluted before the system peak and positive after This response pattern is always obtained when the solute and the probe have opposite charges

Analytes with the same charge as the probe, as well as uncharged solutes, give a reversed response pattern with positive peaks before the system peak and negative after, as demonstrated by a chromatogram given by alcohols in a system with an uncharged compound as the probe (Fig. 3) [6]. The system peaks appear with a *retention* that is dependent only on the properties of the system, but independent of the nature of the analyte, whereas the *direction* changes with the composition of the injected sample. Each mobile phase com-

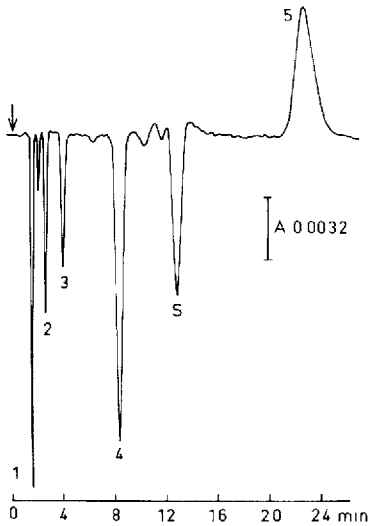

Fig 2 Carboxylic acids Mobile phase, $3 \cdot 10^{-4}$ *M* 1-phenethyl-2-picolinium in acetate buffer (pH 4 6), solid phase, μBondapak Phenyl, detection, 254 nm Peaks 1 = acetic acid, 2 = propionic acid, 3 = butyric acid, 4 = valeric acid, 5 = caproic acid, S = system peak

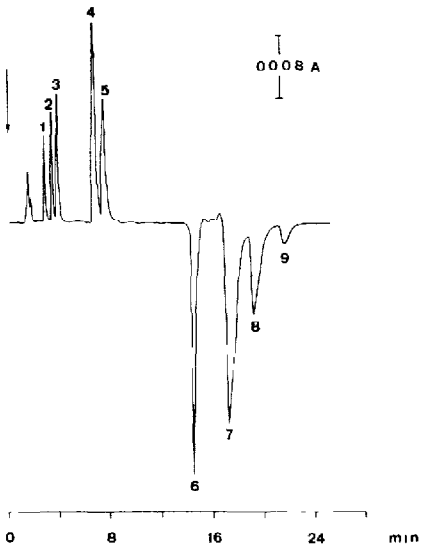

Fig 3 Aliphatic alcohols Mobile phase, 4 10^{-4} *M* nicotinamide in water, solid phase, Ultrasphere ODS, detection, 268 nm Peaks 1 = methanol, 2 = propylene glycol, 3 = ethanol, 4 = 2-propanol, 5 = 1-propanol, 6 = system peak, 7 = 2-butanol, 8 = 2-methyl-1-propanol, 9 = 1-butanol

Fig 4 System peaks Mobile phase, 1 2 10^{-4} *M* benzyltriethylammonium, 1 2·10^{-3} *M* methanesulphonate, 1 1 10^{-3} *M* Na^+ in water, pH 6 1, solid phase, μBondapak Phenyl, detection, 254 nm, sample, water

ponent, except the main solvent, can give rise to a system peak, but the retention will depend on its properties The system peaks can easily be recognized by injection of mobile phase solvent as demonstrated in Fig 4 [7] The mobile phase contains benzyltriethylammonium as cationic probe Injection of water gives a chromatogram with three peaks· the main system peak is strongly re-

tained and negative, whereas the others are positive, only one of them being retained When only one retained system peak appears it represents the probe, which is the most hydrophobic mobile phase component

The simple rules for the response of a detector that is specific for the probe (like the UV detector in the examples above) are summarized in Table 1 [7] They are valid without exception when the mobile phase only contains a hydrophobic probe dissolved in an aqueous buffer with highly hydrophilic components The properties and the amounts of other sample components have no influence on the indirect response in stable chromatographic systems An illustration is given in Fig 5 [7], which shows the response on injection of a mixed sample containing cations and anions of different hydrophobicity in a system with naphthalene-2-sulphonate as anionic probe. Deviations from the simple rules might appear in unstable systems and when the mobile phase contains several retained components, as will be discussed below

TABLE 1

RESPONSE WITH A HYDROPHOBIC DETECTABLE COMPONENT IN THE MOBILE PHASE

k'_s denotes the capacity factor of a hydrophobic detectable component in the mobile phase (the probe) and k'_x the capacity factor of the solute

Charge of solute	Direction of solute peak	
	$k'_x < k'_s$	$k'_x > k'_s$
Opposite to S	Negative	Positive
Same as S or uncharged	Positive	Negative

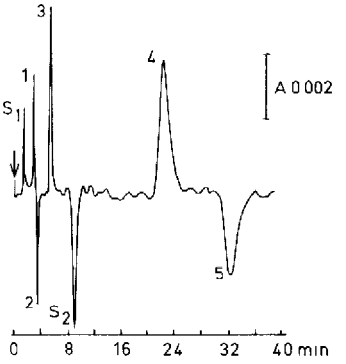

Fig 5 Amines and sulphonates Mobile phase, $4 \cdot 10^{-4}$ *M* naphthalene-2-sulphonate in phosphoric acid (0 05 *M*), solid phase, μBondapak Phenyl, detection, 254 nm Peaks 1 = pentanesulphonate, 2 = diisopropylamine, 3 = hexanesulphonate, 4 = heptylamine, 5 = octanesulphonate, S_1 and S_2 = system peaks

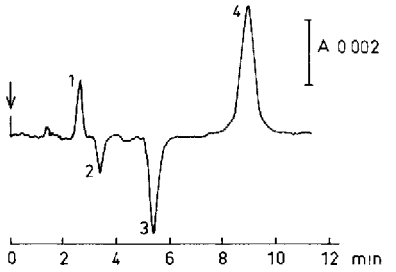

Fig 6 Amino acids Mobile phase, $4 \cdot 10^{-4}$ *M* naphthalene-2-sulphonate in 0 05 *M* phosphoric acid, solid phase, μBondapak Phenyl, detection, 254 nm Peaks 1=DOPA, 2=tyrosine, 3=phenylalanine, 4=system peak

The principles for the indirect response can be summarized as follows The *direction* of a peak originating from a solute depends on the charge and the retention of the solute relative to the probe. It is independent of the injected amount of the solute. The *size* of the peak (the absolute value of the response) is directly proportional to the amount of solute

When a solute has inherent detectable properties the indirect detection phenomena will decrease or increase the observed response, depending on the direction of the indirect effect An example is given in Fig. 6 [5], which shows three amino acids in cationic form as solutes in a system with the highly UV-absorbing naphthalene-2-sulphonate as anionic probe. The solutes are eluted before the system peak where the indirect effect has a negative direction. Tyrosine and phenylalanine, with fairly low inherent molar absorptivity, give negative peaks, i e. the indirect detection effect dominates DOPA, on the other hand, gives a positive peak owing to its very high molar absorptivity, which results in a dominance of the inherent response over the indirect one

3 THEORETICAL BACKGROUND

When a sample is injected into a chromatographic system, the established equilibria between mobile and stationary phase will be disturbed The disturbances will affect all compounds that are coupled by a common interaction effect In reversed-phase systems this common effect usually is a competition for the limited capacity of the adsorbing solid phase Migrating zones are formed for all compounds present in the injection zone: one zone for each injected analyte and one for each mobile phase component, except the solvent. The mobile phase has in these zones another composition than the solution that is pumped into the system, and the zones will appear in the chromatogram as peaks for the solutes and for the mobile phase components (system peaks). It should be remembered that every injection will give rise to such disturbances

but the zones appear as peaks only when the mobile phase contains a detectable component.

The mathematical expressions for retention and response that can be derived on the basis of these general principles are remarkably simple when the mobile phase contains one retained component only. The background has been elucidated by Helfferich and Klein [8], Riedo and Kovats [9] and Melander et al. [10], and applications of the theoretical principles to charged and uncharged solutes have been presented by Crommen and co-workers [11,12] and Schill and Crommen [13]

If a mobile phase component k and an injected solute j, which are both uncharged, compete for the binding surface in accordance with a Langmuir model under analytical conditions (low amounts of injected solute), the capacity ratios are

$$k'_j = AK_j(1-\theta_k) \tag{1}$$

$$k'_k = AK_k(1-\theta_k)^2 \tag{2}$$

θ_k is the fractional coverage of the adsorbent by k, A is the binding capacity of the adsorbent times phase volume ration, and K_k and K_j are the distribution constants of k and j, respectively.

UV absorption has been the dominant technique in the studies on indirect detection, and the expressions below are based on this principle However, it should be emphasized that conclusions drawn from these expressions can be applied to any other specific detection technique for the probe

The detection sensitivity for a solute can be expressed as the apparent molar absorptivity, ϵ^*, which is the peak area, in absorbance units, times the volume per mole of solute injected. On the basis of Langmuir distribution equilibria in the system, it is possible to deduce the following expression for the relative response of the solute j, defined as the quotient of the apparent molar absorptivity to the known molar absorptivity of the probe, ϵ_k.

$$\frac{\epsilon_j^*}{\epsilon_k} = \theta_k \frac{\alpha_s}{1-\alpha_s} \tag{3}$$

$\alpha_s = k'_j/k'_k$, i.e the retention of j relative to k The solute j and the probe k are both uncharged.

Analogous deductions can be made for charged solutes and probes When the solute j and the probe k have the same charge and compete for the adsorbing surface, the relative response can be calculated by eqn. 3. When j and k have opposite charges and promote each other's binding as ion pairs or complexes, the relative response is given by

$$\frac{\epsilon_j^*}{\epsilon_k} = -(1-\theta_k)\frac{\alpha_s}{1-\alpha_s} \tag{4}$$

Eqns 3 and 4 can be used to estimate the relative response of charged solutes when the hydrophilic buffer components in the mobile phase are present in high excess over the probe. When this is not the case, a further term must be added (cf ref 11) However, a high excess of buffer is often a necessity for good stability of the chromatographic system

The equations show that the relative response depends on two factors: the relative retention of the solute (with the probe as reference) and the fractional coverage of the adsorbent by the probe The application of the equations is illustrated in two figures Fig 7 shows a system with naphthalene-2-sulphonate as the probe and the dotted lines give the estimated relative response for cationic and anionic solutes [13] Fig 8 shows the corresponding estimations for uncharged solutes in a system with salicylamide as the probe [12]. The values of θ and $(1-\theta)$ needed for the calculation have been obtained from the linear plot of ϵ_j^*/ϵ_k versus $\alpha_s/(1-\alpha_s)$ (cf eqns. 3 and 4).

Figs 7 and 8 illustrate that the relative response rises from zero to a maximum value in the α_s range 0–1 and then levels out to an almost constant value at higher α_s It should be mentioned that Takeuchi et al. [14] have recently presented an expression for induced solute peak area based on simulation technique. The expression is similar to eqn 3 but it indicates that even unretained uncharged solutes should give an indirect response, which is not in accordance with experimental observations

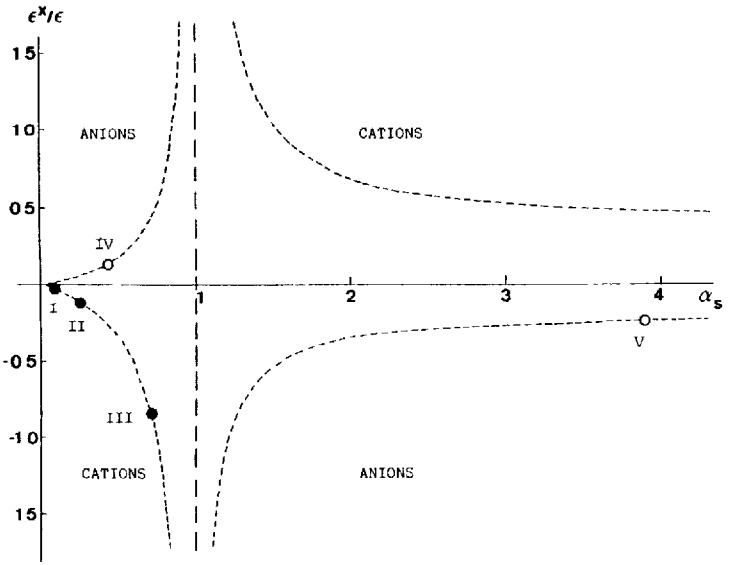

Fig 7 Indirect detection with anionic probe Mobile phase, 10^{-4} *M* naphthalene-2-sulphonate in 0 05 *M* phosphoric acid, solid phase, μBondapak Phenyl, detection, 254 nm I = triethylamine, II = hexylamine, III = tripropylamine, IV = hexanesulphonate, V = octanesulphonate

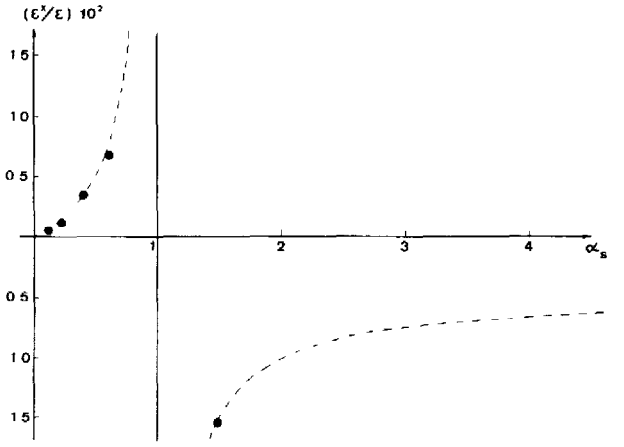

Fig 8 Indirect detection with uncharged probe Mobile phase, $3 \cdot 10^{-4}$ *M* salicylamide in water–methanol (9 1), solid phase, Nucleosil C_{18}, detection, 315 nm Solutes (in increasing retention order) acetonitrile, N,N-dimethylformamide, ethyl formate, 2-butanol, ethyl acetate, 1-pentanol (----) Calculated response

Indirect detection effects can also appear in systems without competition for an adsorbing stationary phase if there are other kinds of common interactions, such as complexation or protolysis

4 DESIGN OF DETECTION SYSTEMS

Optimization of the detection sensitivity should, according to the response equations, be based on three factors the absorptivity of the probe, the fractional loading of the probe on the adsorbent and the relative retention of the solute A high probe absorptivity is obviously favourable but an optimization of the retention in such a way that the solute is eluted close to the probe can be of even higher importance It can be made by adapting the system to the solute by changing the nature and the concentration of the probe as well as the hydrophobicity of the solid phase An approach based on UV detection and suitable for ionic solutes is given in Table 2 [15] The principle is very simple the higher the alkyl content of the solute, the higher the hydrophobicity of the probe and the lower the hydrophobicity of the adsorbent

The chromatographic systems should be as simple and stable as possible. The mobile phase should preferably only contain one retained component, the probe If the mobile phase has such a composition that several retained system peaks are obtained, it can significantly affect the response of the solute Decrease of sensitivity and even a change of the response direction has been reported [15–17] If the solutes are protolytic the mobile phase must contain a buffer with such a capacity that the distribution in the chromatographic sys-

TABLE 2

DETECTION SYSTEMS FOR CATIONS AND ANIONS OF DIFFERENT HYDROPHOBICITY

Probe	Solid phase	Number of CH_2 groups in analyte	
		Alkylamine	Alkylsulphonate
6-Hydroxynaphthalene-2-sulphonate	PRP-1	2–3	5
Naphthalene-2-sulphonate	μBondapak Phenyl	5–7	6–8
1-Phenethyl-2-picolinium	μBondapak Phenyl	7–8	5–6
N,N-Dimethylprotriptyline	Nucleosil CN	9–12	9–12

tem is not affected by pH changes. If the buffer capacity is insufficient, peak distortion and even extra peaks can appear. The buffer component should be hydrophilic and, of course, without inherent detector response. The probe should be aprotic to avoid detection disturbances due to protolysis [17]

Hydrophilic, uncharged additives to the mobile phase might be used in the adaptation of the chromatographic system to the solute as they usually do not give rise to disturbing system peaks It should be noted that such modifiers will decrease the loading of the probe on the adsorbent with decreased detection sensitivity as a result [16,18]. Addition of methanol can, however, have a favourable effect on the peak symmetry and give an increased peak height [18]

It is in principle possible to use any kind of detector that gives sufficient sensitivity and stability The prevailing detection principle has so far been UV absorbance but the use of fluorescence [7,19], electrochemical detection [20], atomic absorption spectroscopy [21], polarimetry [22] and radioactivity [23] has also been reported.

The indirect detection is due to a change in the background signal As the change often is of a magnitude of 1% or less, the chromatographic system must be highly stable and efficient thermostatting of separation column and mobile phase reservoir is necessary The background signal can be rather high: in UV-absorbing systems a background absorbance can be 1 5 or higher and high-quality detectors are needed to avoid disturbing noise and non-linear response [5,15]

5 DETECTION SENSITIVITY

A probe with high molar absorptivity and an adjustment of the relative retention, α_s, to a level close to unity are the main means to attain high detection sensitivity An example is given in Fig 9 [15], which shows the apparent molar absorptivity for a series of non-UV-absorbing anionic and cationic solutes when a cationic probe, N,N-dimethylprotriptyline (molar absorptivity 14 000 at 291

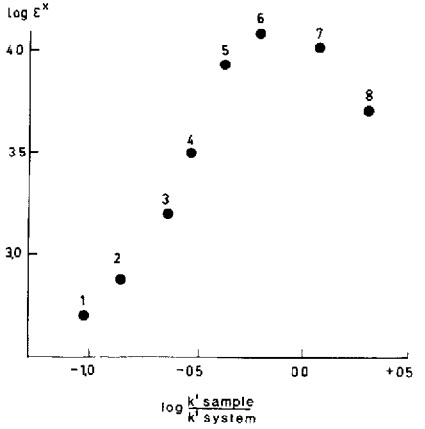

Fig 9 Hydrophobic cationic and anionic compounds Mobile phase, 1 8 10^{-5} *M* N,N-dimethyl-protriptyline in 0 01 *M* acetic acid, solid phase, Nucleosil CN, detection, 292 nm 1 = Octylamine, 2 = octanesulphonate, 3 = octyl sulphate, 4 = decylamine, 5 = tetrabutylammonium, 6 = undecylamine, 7 = dodecylamine, 8 = tetrapentylammonium

TABLE 3

RESPONSE AND RETENTION WITH 1-PHENETHYL-2-PICOLINIUM AS PROBE

Solid phases, μBondapak Phenyl, mobile phases, $3 \cdot 10^{-4}$ *M* 1-phenethyl-2-picolinium (Q) in 0 1 *M* acetic acid ($C_{H+} = 1\,3 \cdot 10^{-3} M$), $k'_Q = 4\,3$, $\epsilon_Q = 3\,03\ 10^3$

Solute	α_S	ϵ^x/ϵ
Dibutylamine	0 28	0 031
Tripropylamine	0 38	0 091
Octylamine	1 27	−1 48
Nonylamine	3 70	−0 52
Decylamine	10 4	−0 53
Methyl sulphate	0 41	−0 11
Propyl sulphate	0 84	−1 41
Butyl sulphate	1 74	0 95
Pentanesulphonate	2 47	0 55
Hexanesulphonate	5 3	0 57

nm [24]) is present as the probe The maximum sensitivity, $\epsilon^* = 12\,000$, is obtained for undecylamine, which is eluted with $\alpha_s = 0\,8$

From eqns 3 and 4 it follows, furthermore, that solutes which are eluted very close to the probe can get a relative response higher than 1 0 in absolute value Experimental confirmation is given in Table 3, which gives results obtained with 1-phenethyl-2-picolinium as UV-absorbing probe [11]

The indirect response also increases according to eqn 3 with the fractional coverage of the adsorbent by the probe, θ, when the solute is uncharged or has

TABLE 4

RESPONSE OF UNCHARGED SOLUTES WITH NAPHTHALENE-2-SULPHONATE AS PROBE

Solid phase, μBondapak Phenyl, mobile phase, $2 \cdot 10^{-4}$ *M* sodium naphthalene-2-sulphonate (NS) in 0 05 *M* phosphoric acid, $k'_{NS} = 8\ 3$, $\epsilon_{NS} = 3\ 00 \cdot 10^3$

Solute	α_S	ϵ^x/ϵ
1-Butanol	0 20	0 005
1-Pentanol	0 55	0 043
Methyl isobutyl ketone	1 15	−0 069
Caproic acid	1 41	−0 057
Hexanol	1 61	−0 038
Butyl acetate	1 93	−0 019
Heptanoic acid	3 97	−0 028
Octanesulphonate	3 72	−0 279

the same charge as the probe Studies on uncharged probes have shown that it is possible to improve the sensitivity by increasing the probe concentration in the mobile phase [12,16] Similar results have not been obtained in ion-pairing systems, possibly owing to the binding to two kinds of sites on the adsorbent [11]

The detection sensitivity for uncharged compounds is much lower than for ionic solutes with similar relative retention in systems with a charged probe [7,11] An example is given in Table 4; the uncharged heptanoic acid has ten times lower relative response than the anionic octanesulphonate with about the same α_s [11] The difference is probably due to differences in the binding to the solid phase The response for uncharged solutes is also rather low in systems with uncharged probe which seems to be due to a low fractional coverage of the adsorbent by the probe [12,18].

6 APPLICATION OF INDIRECT DETECTION IN REVERSED-PHASE SYSTEMS

During the past decade a large number of papers have been published on the indirect detection of organic ions in systems with aqueous mobile phases. One of the first papers on this topic was presented by Parris [25], who used a UV-absorbing quaternary ammonium ion (Hyamine 1622) as a probe for the detection of free and conjugated bile acids Cationic surfactants were detected with aromatic sulphonates as UV-absorbing probes [26] In some of the early papers the indirect detection effect was explained as due to an ion-pairing mechanism (cf refs 27 and 28) whereas others used a more general approach and tried to develop quantitative models by simulation technique (cf ref 29)

The fact that the background to the indirect detection effects could be found

in the work by Helfferich and Klein [8] was not recognized in the early studies on the topic However, the general character of the response effect was indicated by the observations of Slais and Krejci [30] that uncharged compounds could give indirect response in systems with refractive index detection Gnanasambandan and Freiser [31] showed that alkanols could be detected with methylene blue as the UV-absorbing component in the mobile phase

Most of the studies on indirect detection are in fact empirical, but the application of the methods can give results of great practical value if the recommended chromatographic systems have a simple composition with only one hydrophobic component The technique should furthermore not be applied to highly impure or complex samples since the indirect detection is unspecific and response is given by all retained sample components

6 1 Charged solutes

The indirect detection technique has been applied to a wide variety of organic ions in reversed-phase ion-pair chromatography Ionic probes give higher

TABLE 5

INDIRECT DETECTION OF ORGANIC IONS

Analyte	Probe	Mobile phase solvent	Solid phase	Detection wavelength (nm)	Ref
C_5–C_8 Sulphonates C_5–C_{10} Amines	1-Phenethyl-2-picolinium	0 1 *M* Acetic acid	μBondapak Phenyl	254	5
C_2–C_6 Carboxylic acids	1-Phenethyl-2-picolinium	Acetate buffer (pH 4 6)	μBondapak Phenyl	254	5
C_5–C_8 Sulphonates C_5–C_7 Amines Norleucine, phenylalanine, DOPA, tyrosine, leucylserine, leucylalanine	Naphthalene-2-sulphonate	0 05 *M* H_3PO_4	μBondapak Phenyl	254	7
C_8 Sulphonate C_8–C_{12} Amines, tetrabutyl-, tetrapentylammonium	N,N-Dimethylprotriptyline	0 01 *M* Acetic acid	Nucleosil CN	254	15
Formic, butyric, lactic, glycolic acids	$Fe(Phen)_3SO_4$	0 5 m*M* Acetate buffer	PRP-1	511	32
Proline, valine, methionine, ε-aminocaproic acid, tranexamic acid, leucine	DOPA	0 05 *M* H_3PO_4	Ultrasphere ODS	280	6
Tartrate, citrate	Naphthylmethyltributylammonium	10 m*M* Acetate buffer	Supelco LC18DB	254	33

TABLE 6

INDIRECT DETECTION OF NON-ELECTROLYTES

Analyte	Probe	Mobile phase solvent	Solid phase	Detection wavelength (nm)	Ref
Alkanol, alkyl ester, dimethylformamide	Nicotinamide	Water	Nucleosil C_{18}	263	18
Alkanol, alkyl ester, dimethylformamide	Salicylamide	Water–methanol (9 1)	Nucleosil C_{18}	315	12
C_5–C_9 Alkanes	Anthracene	Acetonitrile–water (75 25)	ODS SC-01	250	35
C_5–C_9 Alkanes	Chrysene	Acetonitrile–water (75 25)	ODS SC-01	269	35
Poly(ethyleneglycol)	Caffeine or Theophylline	Acetonitrile–water (9 1)	ODS SC-01	210	36
Alkanol (in liquors)	Theophylline	Methanol–water (12 88)	Develosil ODS3K	210	37

detection sensitivity than uncharged probes, as discussed above, and the probe can serve as counter-ion or competing ion to the solute The optimization of the relative retention, $\alpha_s = k'_{solute}/k'_{probe}$, to a level close to unity is often more easily made if the probe has the same charge as the solute. A suitable retention can then be achieved by altering type and concentration of the counter-ion

Aprotic probes, e g. quaternary ammonium ions or sulphonates, are often preferred If a protolytic probe is used, the mobile phase must have such a pH and such a buffer capacity that pH changes in the migrating zones do not give protolysis of the probe. Some examples of systems that give good sensitivity under conditions that approach the optimum are given in Table 5

The indirect detection technique can also be applied in ion-exchange systems. If the probe is the only mobile phase component with the same charge as the solute, the fractional coverage will approach unity All solutes will then give negative peaks with an apparent molar absorptivity that is approximately the same as that of the probe [11]. Systems based on this principle are used in ion chromatography of hydrophilic organic ions [cf. 34] The view of the background to the indirect detection effects presented in this review is based on studies in reversed-phase systems. Only limited applications of the principles to other kinds of chromatographic system have so far been made.

6 2 *Uncharged solutes*

Indirect detection of non-electrolytes can be performed with ionic as well as with uncharged probes [5,6,18,31], but the apparent molar absorptivity is considerably lower than that obtained with ionic solutes in ion-pairing systems However, Takeuchi and Ishii [35,36] and Takeuchi et al [37] have shown that a considerable improvement of the mass sensitivity can be achieved by applying the technique in packed micro-bore columns Examples of detection systems that have been studied in greater detail are given in Table 6

An uncharged probe that gives a fairly low apparent molar absorptivity can be favourable in certain cases of impurity testing The width of the main component peak and the system peak will be smaller than with a charged probe, which gives more space in the chromatogram for detection of impurities without inherent response.

7 INDIRECT DETECTION IN SYSTEMS WITH NON-POLAR MOBILE PHASES

The indirect detection technique can also be applied in systems with a hydrophilic adsorbent and a mobile phase with low polarity. An example is given in Fig 10, which shows the separation of the antipodes of two enantiomeric

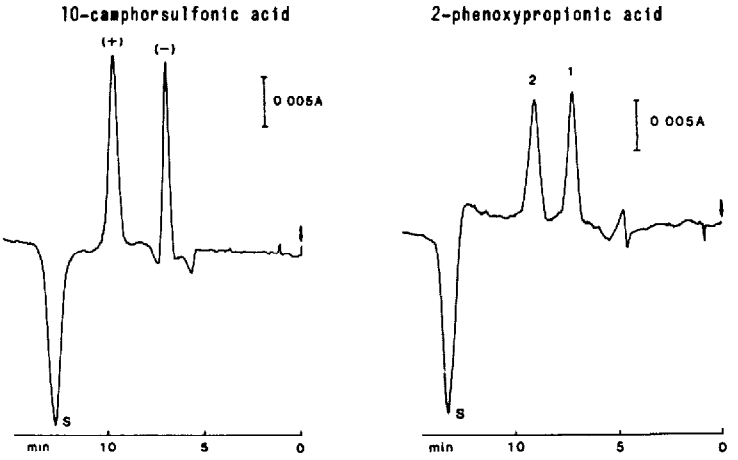

Fig 10 Resolution of (±)-2-phenoxypropionic acid (right) and (±)-10-camphorsulphonic acid (left) Mobile phase, $3\,5 \cdot 10^{-4}$ *M* quinine and $3\,5\ 10^{-4}$ *M* acetic acid in dichloromethane–1-pentanol (199 1), solid phase, Lichrosorb DIOL, detection, 337 nm S = system peak

acids with quinine as the chiral selector [38] The analytes have fairly low molar absorptivity whereas the selector (quinine) has a very high absorptivity at the detection wavelength and will act as a probe The two antipodes of the acids appear as well separated peaks in front of the negative system peak Notice that the peaks of the two antipodes are of unequal size The relative response increases with increasing relative retention, giving the antipode eluted closer to the negative system peak a higher relative response than the first eluted one

8 INDIRECT DETECTION IN BIOLOGICAL SYSTEMS AND OTHER COMPLEX MATRICES

The indirect detection technique is unspecific and it is less suitable for highly complex or impure samples. Unpurified biological samples, e.g urine, contain a large number of mainly hydrophilic compounds of endogenous origin and only hydrophobic solutes can be detected and quantified in such matrices (cf. ref 15) The improved understanding of the background to the indirect detection technique has widened its applicability in biological analysis However, highly hydrophilic solutes will always give problems since the systems then must have such a design that even normal buffer components may give rise to disturbing system peaks (cf ref. 39)

Analyses in pharmaceutical matrices can be simplified by the indirect detection technique Downey and Jenke [40] have quantified oxalate and citrate by ion chromatography with phthalate as probe Crommen and Herné [16] have presented a method for the determination of the divalent methylmethionine sulphonium cation (vitamin U) and its degradation product methionine in solutions for injection Nicotinamide in cationic form was used as probe and the retention of the analytes was regulated by octanesulphonate

Choline, acetylcholine and other choline esters can be analysed in plant extracts by an indirect detection technique with 1-phenethyl-2-picolinium as probe in a reversed-phase system with an acidic mobile phase [41] Aliphatic alcohols have been determined in liquors by an indirect detection technique using theophylline as probe in a reversed-phase system with a packed microbore column [37]

9 DISTURBING INDIRECT DETECTION EFFECTS

Commonly used mobile phase components can under certain conditions give a detector response and cause disturbing or unexpected effects Typical examples are alcohols, such as methanol and propanol, which absorb at wavelengths lower than 250 nm, and tetrahydrofuran, which has a significant absorbance below 280 nm

If such a solvent is present in the mobile phase and the detection is made in

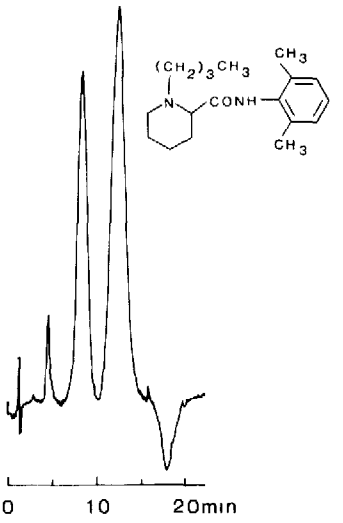

Fig 11 Disturbance by UV-absorbing mobile phase Mobile phase, 8% 2-propanol in phosphate buffer (pH 7 2), solid phase, EnantioPac LKB, detection, 215 nm, solute, bupivacaine (racemate)

the absorbing region, solutes with inherent absorptivity will give a response that changes with the retention An example is given in Fig 11 [13] A racemic compound was separated into its antipodes on a chiral adsorbent with a mobile phase containing 2-propanol, using 215 nm as the detection wavelength. The solute contained the antipodes in equal amounts but in the chromatogram the two peaks have different areas, the more retained being significantly larger The observed response is under these conditions a combination of inherent response and indirect response, and the latter part increases with increasing retention up to the system peak.

The chromatogram contains one positive and one negative extra peak Such extra peaks, which appear when the mobile phase gives a detector response, are usually system peaks. When a negative peak appears in a chromatogram, an indirect detection disturbance should always be suspected. However, such phenomena are often overlooked If the spectra of the two antipodes are taken with the mobile phase as reference, none of the peaks will have the normal spectrum of the solute, which adds to the confusion

The problems are even more difficult if the mobile phase contains an unknown impurity, as demonstrated in Fig 12 [42] A plasma sample is injected into a system with methanol–phosphate buffer as mobile phase Indirect detection effects should not appear as the detection wavelength is 254 nm Two strongly retained peaks (one positive and one negative) appear in the chromatogram. An indirect detection effect was suspected owing to the negative peak, and it was found that the two last peaks disappeared when the mobile phase contained purer methanol A comparison of the two chromatograms

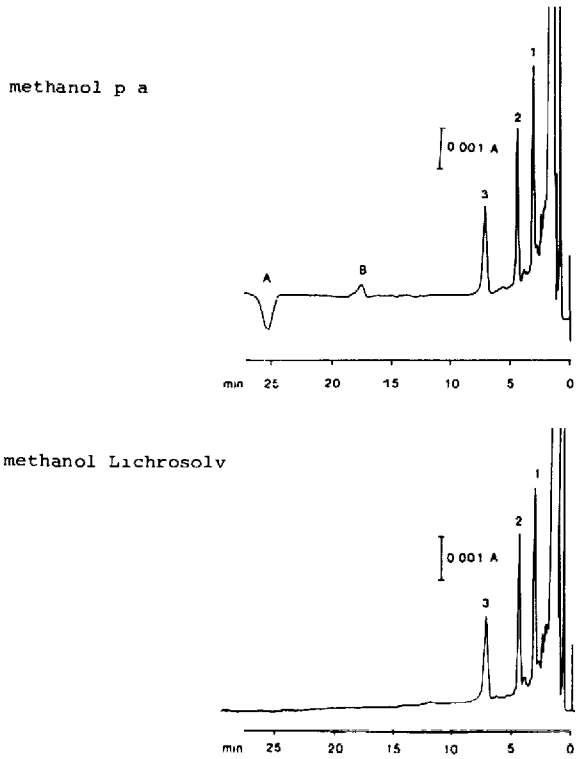

Fig 12 System peaks from mobile phase impurities Mobile phases, (upper chromatogram) methanol p a -phosphate buffer (pH 6 1) (1 1), (lower chromatogram) LiChrosolv methanol-phosphate buffer (pH 5 9) (1 1), solid phase, Nucleosil C_{18}, detection, 254 nm Solutes 1 = oxyphenbutazone, 2 = γ-hydroxyphenylbutazone, 3 = phenylbutazone

shows, furthermore, that the height relationship between solute peaks 1 and 2 has been changed in the presence of the impure methanol

Particularly large system peaks appear if the mobile phase gives a detector response and the injected sample is dissolved in another solvent [5,7,16] Such effects can appear by column switching when the eluate from one column is applied on another column with a different mobile phase

10 CONCLUSIONS ON SUITABLE BIOMEDICAL APPLICATIONS

Indirect detection is a technically simple and sensitive method for detection of compounds without inherent detector response The detection is unspecific and it is especially suitable for samples with a limited number of components, e g for determinations in pharmaceutical products and quantification of impurities or breakdown products in substances The understanding of the underlying principle is essential not only for optimizing the detection sensitivity

but also for avoiding disturbances and unexpected effects arising from mobile phase components with a detector response.

11 SUMMARY

Indirect detection is a technically simple method to follow and quantify compounds without inherent detector response in high-performance liquid chromatography. A detectable component is added to the mobile phase and peaks are obtained for injected solutes as well as for mobile phase additives (system peaks) The underlying principle can be expressed by simple equations which show how the detection sensitivity can be optimized.

The detection technique can be applied to uncharged as well as charged compounds, but the sensitivity is considerably higher for ionic solutes The response is unspecific and the technique should preferably be applied to samples with a limited number of components. Determinations in pharmaceutical products and quantification of impurities in substances are typical fields of application Different detection principles can be used but UV absorbance is so far the dominant technique The chromatographic system must be stable, and efficient thermostatting is essential

Indirect response effects can give rise to disturbances when the presence of a detectable component in the mobile phase is unknown or overlooked The understanding of the underlying principle for the indirect detection is essential for tracing or preventing such disturbances

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