Fresenius J Anal Chem (2000) 366 : 635–645 © Springer-Verlag 2000

REVIEW

Michael G. Weller

Immunochromatographic techniques – a critical review

Received: 7 September 1999 / Revised: 4 November 1999 / Acepted: 8 November 1999

Abstract Hyphenated techniques have become very popular during the last decade. Nevertheless, the use of biochemical methods, such as immunoassays, in conjunction with instrumental methods, such as chromatography, have not gained widespread acceptance. This review critically discusses many of the implemented and potential options for such coupled systems or components, which might be useful for such systems, including immunoaffinity extraction, immunoaffinity chromatography, immunochemical detectors, immunoblotting, receptor assays, enzyme inhibition assays, displacement assays, flow-injection immunoassays, miniaturized techniques and stationary phases such as restricted access materials or molecularly imprinted polymers. The performance of immunochromatographic systems is discussed regarding their ability to solve highly complex and demanding analytical problems.

1 Preface

Immunoassays and chromatographic techniques have long been considered to be competitors. Today it is clear that this view is far too simple. More and more techniques are being developed which combine the advantages of both approaches in highly powerful analytical tools. Nevertheless, the application of such hyphenated techniques is not yet very widespread. This review does not claim to be comprehensive concerning citation, as some reviews covering similar topics have been published already [1–7], but has the aim to inform a broader readership of some especially interesting or exemplary developments in this field and to give an overview of immunochromatographic and related techniques.

Dedicated to the memory of Prof. H. Michael Widmer

M. G. Weller (✉)
Institute of Hydrochemistry, Technical University of Munich,
Marchioninistrasse 17, 81377 München, Germany
e-mail: michael.weller@ch.tum.de

Many immunological techniques exist which use some chromatographic components. Most of them will be described here, at least briefly – irrespective of whether they are in fact true immunochromatographic techniques or only similar techniques. For the sake of completeness, it seemed to be helpful to use neither the definition of immunoassays nor of chromatographic techniques in a strict sense in this review.

2 Why combine immunoassays and chromatography?

Both techniques have their distinctive strengths and drawbacks. Immunoassays are fast, inexpensive and extremely sensitive. However, there is no easy possibility of distinguishing between several cross-reactants. As long as the analyte identity cannot be confirmed by additional information, no real quantification is possible, only “equivalent” concentrations are obtained. Although a group of analytes can be determined as a sum parameter, a real multianalyte analysis is not possible with simple immunoassays. Recently, a system based on a highly parallel biochip has been presented, which may overcome these limitations [8–10]. Nevertheless, additional approaches would be highly desirable. Standard chromatography, however, shows a very high performance for quantitative analysis. Unfortunately, this has to be paid for with high investments and costs per sample and often sufficient sensitivity can only be obtained with extended enrichment procedures. In addition, setup, calibration, enrichment, clean-up and sequential analysis by chromatography generally require much time – in many cases several days pass by, until a result can be given. For both methods it is highly challenging to analyze unknown analytes – especially when standard compounds are not available. Furthermore, the final interpretation of an analytical result remains difficult, if not impossible, e.g., if you need to guarantee the absence of toxic compounds in a sample.

The combination of immunoassays with chromatographic techniques is very effective as the data are essentially orthogonal, which means that there is almost no re-

dundancy, which is often the case when similar techniques are hyphenated. Therefore, one gets much more information about an unknown sample than from one of the techniques alone. Only techniques which use information from both – immunoassay and chromatography – should be considered to be genuine immunochromatographic techniques.

Most immunochromatographic techniques[1] can be classified into two types, the pre-column[2] techniques, such as immunoaffinity extraction (IAE), where antibodies are used as sample clean-up and enrichment reagents, and the post-column techniques, such as immunochemical detectors (ICD), where antibodies are used in an immunoassay mode.

3 Immunoaffinity extraction (IAE)

One of the most well-known techniques in this context is immunoaffinity extraction [6, 11]. There are many synonyms for this technique, such as immunoaffinity chromatography, immunoenrichment, immunoextraction, immunoaffinity enrichment, immunoadsorption and others. The most frequently used term is immunoaffinity chromatography, which is often regarded as the superordinate concept [6]. Although historically based, this term seems to be somewhat misleading. In most cases, antibody-containing columns are not used as in chromatography, but more as in solid-phase extraction (SPE) [12]. Therefore, the analogous term immunoaffinity extraction (IAE) seems to be preferable. In Fig. 1 the general principle of immunoaffinity extraction is shown. A column with immobilized antibodies is loaded with the sample in the first step. The analytes and cross-reactants are bound to the column. It has to be ensured that the binding capacity of the cartridge is not overloaded. With a mild washing step all weakly bound material (matrix molecules, irrelevant analytes, etc.) is eluted from the cartridge and discarded. Finally, the analytes are eluted cautiously with a solvent/water mixture or an acidic buffer. Careful elution is necessary to prevent the denaturation of the antibodies to enable a reuse of the expensive cartridge. Should regeneration of the cartridge not be necessary, much cruder methods can be used for elution. In most cases the extract is directly suitable for HPLC, other separation techniques or even for direct analysis.

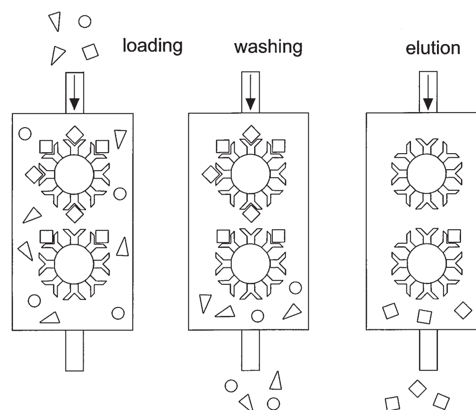

Fig. 1 General principle of immunoaffinity enrichment (IAE). A cartridge with immobilized antibodies (Y-shaped molecules) is used for the separation of analytes (square-shaped molecules) from unwanted matrix components

3.1 Off-line coupling

One important advantage of immunoaffinity extraction is the fact that no expensive equipment is needed, assuming that a standard chromatographic system is available in the lab. Analysts using SPE get to like IAE very soon, as the application is very similar. This may be the reason why IAE has gained increasing popularity over the years [13]. In some cases standard SPE (C18 material) and immunoaffinity extraction were performed successively with encouraging results [14]. Even some commercial products are now available, e.g., for the analysis of mycotoxins (aflatoxins, zearalenon, ochratoxin A, fumonisin and others [15]). Recently, the combination of several monoclonal antibodies to multi-immunoaffinity columns was shown; a useful approach to achieve a broad group selectivity [16]. In most other hapten-immunoassay techniques it is not advisable to mix antibodies.

3.2 On-line coupling

One step more sophisticated is on-line immunoaffinity extraction, which can be implemented mainly with standard HPLC equipment. The disadvantage is that frequent regeneration of the immuno-columns has to be guaranteed. For off-line applications it is possible to use a fresh cartridge for each sample. It should be noted that for this setup the immunocartridge is back-flushed for elution, which is preferable to obtain a smaller elution volume. In cases where regeneration is not possible or not desirable (cross-contamination or reproducibility problems), a throw-away solution is preferable. Although robotic systems are available which facilitate the changing of the solid-phase columns [17], to my knowledge no corresponding immunoaffinity columns are available, yet. Many applications of IAE/LC couplings have been shown (e.g., [18]), even some quite complex applications have been published [19, 20], for instance an on-line IAE/LC/LC/MS/MS coupling for LSD analogues (Fig. 2) and dibenzodiazepines, respectively, where in the first LC step a C18 material or RAM (restricted access material), in the second a conventional C8 or C18 material was used.

[1] Techniques which use immobilized antibodies for a chromatographic separation of several analytes are rare

[2] The term column is used here in the sense of a conventional HPLC column

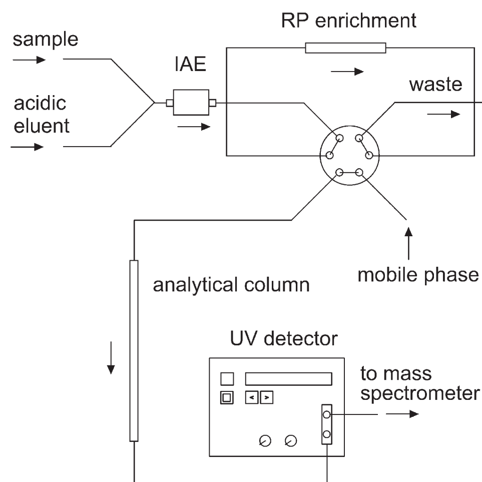

Fig. 2 IAE/LC/LC/MS/MS coupling according to [19]

4 Immunoaffinity chromatography (IAC)

This term is often used in another sense (see IAE). However, there are some techniques which might be denoted as true immunoaffinity chromatography, also known as weak-affinity chromatography (WAC) [21, 22]. First of all it is surprising that this approach – a chromatographic separation on immobilized antibodies – is rarely used. Considering chromatographic theory, one might suspect that the establishment of equilibrium is too slow with antibodies. It is well-known from immunoassays that antibodies need minutes, if not hours, to reach equilibrium. The dissociation process, in particular, is very slow for high-affinity antibodies. Therefore, one can expect a very poor separation efficiency – a very low number of theoretical plates – which in fact was experimentally found. Hence, one can conclude that interactions of low strength are more useful in chromatography. Taking this into account, only weakly binding antibodies should be considered for this purpose. In addition, significant heterogeneity of affinities is found with polyclonal antibodies, which is further increased by the immobilization process. This is why even with monoclonal antibodies binding sites of different affinity result after immobilization. These factors might altogether be the reason for the moderate performance of such separations. Nevertheless, a work was published where immobilized haptens successfully were used for the separation of antibodies of a polyclonal antiserum [23]. One of the tricks seems to be the selection of a hapten with low cross-reactivity and the preparation of a support with low hapten density to ensure an essentially monovalent binding mode. An impressive separation of many antibody fractions was possible and proves the significance of this approach. The problem might be the selection of a suitable hapten.

Perhaps the most frequently used variant of IAC (please note the above definition) is not a true immunotechnique, but based on molecular imprinted polymers (MIPs), which can be considered an antibody substitute (see also section 10.3). The problem with many MIPs which relates to low affinity is an advantage for a chromatographic application. The separation efficiency is quite low in many cases. Nevertheless, useful chromatographic separations of enantiomers are possible, which seem to be the main applications of MIPs at the present time [24–26]. The problem of MIPs that there is often a strong nonspecific interaction of the analytes with the blank-polymer, does not do very much harm in this respect, as both enantiomers are affected identically. Even the preparation of enantioselective thin-layer plates (for TLC) has been described [27]. Another advantage of MIPs in relation to other enantioselective materials is that the elution order can be predicted very easily (the imprinted enantiomer will elute later).

5 Immunochemical detectors (ICD)

5.1 Labeled reagents

Quite a recent development are immunochemical detectors, which can be implemented in on-line or off-line setups. Off-line detection especially has been used with increasing frequency, as more and more powerful immunoassays have become available. In the simplest form, the eluate of a HPLC system will be divided into equal fractions. These will be subsequently analyzed by immunoassay or a similar technique. This approach was successfully used for the analysis of cyanobacterial toxins in natural freshwater [28]. The most difficult problems are the optimization of the sensitivity, which can be done for instance by dilution or variation of the injection volume, and the interfering effect of the organic solvents in the mobile phase, which also can be avoided by sufficient dilution of the eluent. Competitive assays have the additional problem that their working range is only about 2–3 decades in concentration. Therefore, it is relatively difficult to find a suitable dilution factor. Antibodies of extreme affinity are preferable not only for standard immunoassays, but also for many immunochromatographic techniques (except IAC). When a sufficiently high dilution factor can be chosen, no solvent interference will remain. Finally, almost all other matrix-dependent problems in immunoassays can also be solved with dilution. The reasons why assays with labeled reagents are largely preferred, despite their fundamental disadvantages, are their selectivity and sensitivity. Assays working with non-labeled compounds are very vulnerable to matrix effects and often are not sensitive enough as any enhancement mechanism (for instance by enzymes) is lacking.

5.1.1 Labeled antibodies

On-line immunochemical detectors have rarely been used up to now. These detectors also could be considered to be immunosensors [29] or biosensors [30], respectively. Irth

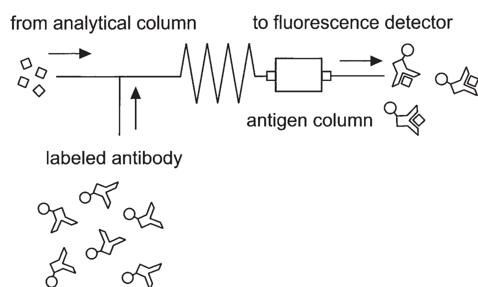

Fig. 3 Immunochemical detector with labeled antibodies – separation by antigen column (see also Fig. 7). The circles symbolize a label, for instance a fluorescence dye

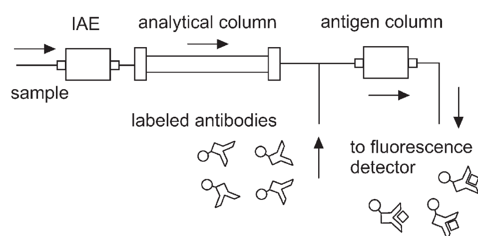

Fig. 4 Combination of immunoaffinity extraction (IAE) and immunochemical detector (ICD) with labeled antibodies [33]. The analytical column is filled with a reversed phase (RP) material

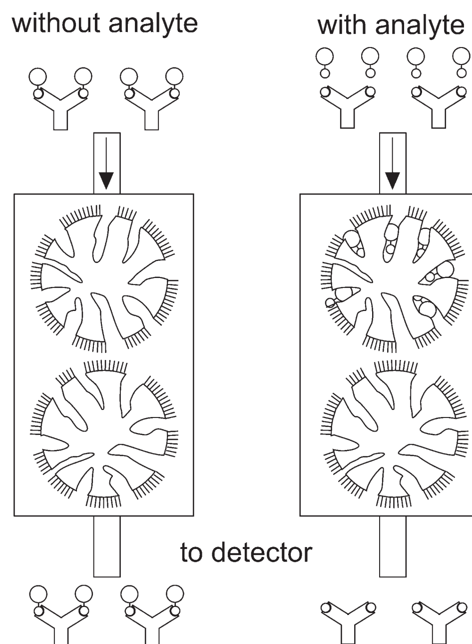

Fig. 5 Immunochemical detector with labeled haptens – separation by restricted access material (RAM). In the case of present analyte, the free fluorescence tracer (large circles: fluorescence dye, small circles: analyte) will bind to the RAM column, and therefore cannot reach the detector

et al. published an on-line HPLC-ICD with labeled antibodies [31, 32]. The system is similar to the non-competitive immunoassay, which is described in section 7.6. The eluate of the HPLC is mixed post-column with fluorescence-labeled antibodies (Fig. 3). After passing a reaction coil, the mixture is pumped through a column filled with an immobilized antigen (or hapten). All free antibodies will stick quasi-irreversibly on this column. Only antibodies (or Fab fragments) with bound analyte can pass the column and are quantified with a HPLC-fluorescence detector. The advantage of this elegant system are the fast kinetics, which is caused by the surplus of labeled antibodies. In addition, the signal is proportional to the analyte concentration, which is generally not the case in other hapten immunoassays. A significant drawback seems to be the labeled antibody reagent. The antibody (fragment) has to be extremely pure as all kinds of labeled impurities lead to high background signals. It should be noted that even partially or fully denatured antibodies – they are not retained by the antigen column any more – contribute to this background. In addition, the manufacturing of the antigen column and of the labeled antibodies may be quite costly. However, the system is a highly selective and sensitive detector, which may be tailored to all kinds of analytes for which suitable antibodies are available. Even an IAE-LC-ICD hyphenation (Fig. 4) has been implemented based on this type of detector [33].

5.1.2 Labeled haptens

To avoid the critical labeling of antibodies, an inverse system was developed [34], which used labeled analyte (hapten). Instead of using expensive immobilized antibodies, the unbound fluorescence-labeled hapten was bound by a column filled with a restricted access material (RAM, Fig. 5). Only the small hapten conjugate is bound, not the hapten-conjugate/antibody complex. First, the eluate of the HPLC is mixed post-column with antibody, then with the fluorescence tracer. In the case of no analyte present, most of the fluorescence tracer will bind to the antibodies and as this tracer/antibody complex will not bind to the RAM, a maximum of dye will appear at the fluorescence detector. With increasing analyte concentration, more and more fluorescence tracer will be left free and subsequently bound to the RAM, and a decrease in fluorescence intensity will be noticed at the fluorescence detector. In contrast to the system mentioned above, this is a competitive assay and shows an inhibition of a strong signal. The problems with labeled antibodies are avoided, but other problems may become more prevalent. First of all, the kinetics are not as fast, as higher antibody concentrations always have to be paid for with reduced sensitivity, as in all competitive assays. In addition, the inhibition design limits the working range largely to two or three decades, which is not much for a detector. Advantages are the use of relatively cheap RAM, which does not have to be regenerated very often, as the capacity of the material is very high. The hyphenation of a RAM in on-line sample enrichment and clean-up with a fluorescence tracer/RAM immunochemical detector was demonstrated (Fig. 6, [34]). Finally, non-chromatographic applications of RAM immunoassays have been shown [35].

The on-line application of a hollow-fiber system was shown to be an additional method to separate bound and

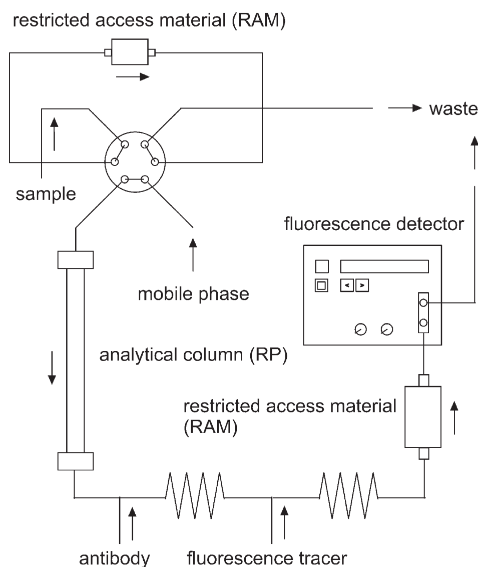

Fig. 6 On-line system [34] using an immunochemical detector (ICD) with labeled haptens (tracer)

free labeled analytes [36]. The first part of the immunochemical detector is very similar to the previous one. The antibody is mixed with the HPLC-eluate post-column and subsequently, after a reaction coil, with the fluorescence tracer (fluorescence dye/hapten conjugate). After a further reaction coil the mixture passes the separation module, which consists mainly of a porous hollow fiber. In contrast to the system discussed above, where the antibody/tracer complex was measured in the fluorescence detector, here the antibody/tracer complex is directed to waste, only the free tracer, which passed the hollow fiber wall is detected. The advantage is that, similar to the antibody-labeled system, an analyte-proportional signal is obtained. Unfortunately, the sensitivity and separation efficiency of the system seem to be relatively poor.

5.2 Label-free methods

Label-free methods have many advantages [37]. Nevertheless, in this context only very few examples have been reported up to now. Label-free immunological methods can also be considered to be biosensors. Systems based on piezoelectric microbalances, surface acoustic wave sensors, lamb wave sensors, interferometric techniques, surface-plasmon resonance, resonant mirror, bioforce sensors, microcalorimeters, biological sensors (receptors) may be possible. One of the problems seems to be the small time constant, which is necessary for high-resolution chromatography. Many of the sensors have quite an acceptable response time, but need very long to recover. In addition, the sensitivity seems to be worse as in labeled systems. Furthermore, many of the systems may have considerable problems to cope with a non-constant refractive index or pH, which may vary during a chromatographic run in a gradient system. As there are many points in favor of gradient separations (speed, higher efficiency, higher sensitivity, etc.) a useful immunochemical detector should be suitable for gradient systems, also. One of the first attempts was published in 1994 [38]. The "off-line detector" was based on a commercial BIAcore system (surface-plasmon resonance). An early on-line system using a surface acoustic wave detector was reported in 1995 [39]. For special analytes their intrinsic fluorescence can be used. Hence, the detection of polycyclic aromatic hydrocarbons (PAHs) was achieved with a fluorescence sensor based on MIPs [40]. However, there is no attempt known up to now that this principle has been coupled to a chromatographic system.

6 Indirect hyphenation

Recently, a capillary electrochromatography system (CEC) with reversed phase (C4) preconcentration was presented for the analysis of steroids [41]. The connection to immunological methods was achieved by the addition of particles with immobilized antibodies to the sample. The measurements of a raw sample and the sample with affinity particle treatment showed significant differences. The peaks which belong to the antibody-bound species are lacking in the second electrochromatogram. Thus, the immuno-active substances can easily be assigned. For confirmation purposes, the immunoparticles can be extracted and the eluate examined similarly to IAE.

7 Pseudo-immunochromatographic methods

As mentioned before, only methods using information from both the immunological and the chromatographic step, should be denoted immunochromatographic techniques. In this section some other methods are discussed, which have some superficial similarity with immunochromatography. The term pseudo has by no means to be understood as a negative valuation.

7.1 Immunofiltration

This term may imply similarities to immunoaffinity extraction IAE. However, immunofiltration can be regarded as an own immunoassay format. In contrast to a microtitration plate (MTP), where the volume of sample is limited to about 200 µL, the sample is filtered through a porous membrane [42]. The antibodies are immobilized on this membrane. Afterwards, the membrane is subjected to a usual competitive immunoassay with colorimetric detection. Sometimes it is suspected that the analyte is enriched on the antibodies on the membrane and that therefore lower detection limits can be achieved in relation to standard MTP formats [43]. It seems that unquestionable proof is still lacking, as in most papers only one method is shown. It has to be kept in mind that – at least competitive assays – are often affinity-limited, and therefore nearly all

assay formats using the same antibody should lead to essentially the same detection limit, which could be shown experimentally [9] and by simulation [44]. Different detection limits are therefore caused by a different optimization intensity. The problem of the above reasoning is that if you use a large amount of antibodies to improve the enrichment, the competition step is hampered. If you use a low amount of antibodies, then the enrichment process will soon reach equilibrium, and the rest of the sample volume will break though without any further enrichment. Of course, immunofiltration is no chromatographic method, but it can be useful for fast on-site analysis without any technical equipment.

7.2 Tracer presaturation assays

Tracer presaturation assays, often denoted displacement assays or displacement flow immunosensors, are competitive immunoassays usually for small compounds (haptens). Their special feature is the saturation of the immobilized antibodies with a labeled hapten (tracer). In the analysis step the sample is introduced – without any additional reagents. The displaced tracer will be detected. In the recent years several papers have been published in this field, e.g., in [45]. Nevertheless, it was surprising that some of the known immunoassay formats could be performed as tracer presaturation assays, but most assays do not work if the antibodies are presaturated with tracer. Considering that immunoassay theory often assumes that equilibrium is obtained, this result is strange. Per definition it is irrelevant from which side equilibrium is established. Upon closer consideration one can see that all successful setups use a column-like system, as in chromatography. Analyzing the potential mechanism, one can conclude that tracer presaturation assays are in fact non-equilibrium systems. All such assays probably do not rely on true displacement but on dynamic competition. In general, the concentration of a free analyte should not influence the dissociation rate. It is much more likely that displacement assays are based on a slow leaching of tracer, which moves down the immunoassay column. If the entire column was presaturated, this leads to a constant background signal – which can in fact be seen in many of the corresponding papers. Injecting a sample which contains some analyte, this analyte will bind to free binding sites at the column and therefore inhibits the rebinding of the leaching tracer. Therefore, one observes a signal peak of the eluted tracer which is proportional to the amount of the injected analyte. As there is no evidence that a displacement mechanism is involved in these assays, this type should better be denoted tracer presaturation assay.

7.3 Immunochromatographic dipsticks

These test strips are based on a capillary migration of a reagent mixture [46]. They have indeed some mechanistic similarity to the tracer presaturation assays, discussed in the previous section, as analyte molecules, which bind to immobilized antibodies, block these sites and thus influence the migration of the tracer. As more analyte means enhanced tracer migration, the resulting color band will move higher. This can be used in a thermometer-like setup, which is very useful especially for unskilled users.

7.4 Multianalyte dipsticks

A so-called lateral flow device or immunomigration strip is the basis for these assays. Often gold particles or liposomes [47] are used as a tracer as they do not need an enzymatic enhancement for visual detection. It is possible to manufacture test-sticks with several visible reaction zones, e.g., for the detection of several drugs of abuse in urine [48]. Although these bands have some similarity to chromatographic bands in TLC, they are caused by a completely different mechanism. The positions of the zones do not move, they are predefined by the immobilization of different reagents at selected zones.

7.5 Flow-injection immunoassays (FIIA)

In the frequency of application of immunochromatographic techniques, the FIIA probably comes second after the IAE. In this respect an immuno-column is used, also. In contrast to IAE, where the antibodies are only used to enrich and/or to clean up the analyte to be transferred to another analytical device, in FIIA a full immunoassay is performed on the immuno-column [49–51]. In the simplest form, the antibodies or antigens are directly immobilized on the column. The problem is that antibodies are easily denatured, especially during the regeneration cycle [52]. Therefore, these assays seem to depend in some respect on a surplus of antibodies on the column, which successively lose activity. To reduce these problems, quite often protein A [53] or protein G [54] columns are used. Protein A (or G) selectively binds antibodies (IgG of many species and subclasses). With weakly acidic buffers, the antibodies can be eluted without destroying the activity of the protein A/G column. In this setup the antibodies are freshly immobilized on the protein A/G column with each cycle. The disadvantages are that the recoating procedure needs additional time and consumes significant amounts of relatively expensive antibodies. On the positive side, you have almost no regeneration problems and quite reproducible signals with good sensitivities. In this biosensor type the column is only used for separating bound and free labeled reagents. It is possible to monitor the amount of labeled hapten directly on the protein A column of a flow-through sensor by fluorescence spectroscopy [55]. Detection limits in the lower µg/L range were achieved for the herbicide atrazine.

7.6 Non-competitive immunoassays for haptens

Some years ago, it was common knowledge that for haptens (small molecules) it is necessary to perform competitive immunoassays, which have some physicochemical disadvantages in contrast to non-competitive assays. For instance, competitive assays are always affinity limited – assuming that a sufficiently sensitive detection system is available. The possibility to detect chemically reactive analytes in a non-competitive assay [56] will not be discussed here any further. Interestingly, another technique has been developed [57, 58], which overcomes the affinity limitation, at least in some aspects. This assay is based on the use of hapten columns (Fig. 7). Although this has not been examined in detail, one can assume that this assay is dissociation-limited, not affinity-limited. However, one has to keep in mind that in most cases the affinity constant and the dissociation rate constant are inversely proportional to one another. One of the most obvious advantages seems to be the accelerated association kinetics, which are caused by the higher antibody concentration. Therefore, very fast immunoassays with a high sensitivity are possible. A clear disadvantage is the high consumption of expensive antibodies. In this assay format, also, the hapten column is only used to separate two species, the free antibody from the antibody-hapten complex. It should be noted that the cross-reactivities in such a non-competitive assay are completely different to those in a competitive assay [56]. In addition, the bivalency of IgG is especially undesirable in this respect, and therefore Fab fragments or similar monovalent antibodies are used preferentially. Although the non-competitive assay described is not a true immunochromatographic technique, it has been used for the construction of a immunodetector (see section 5.1.1).

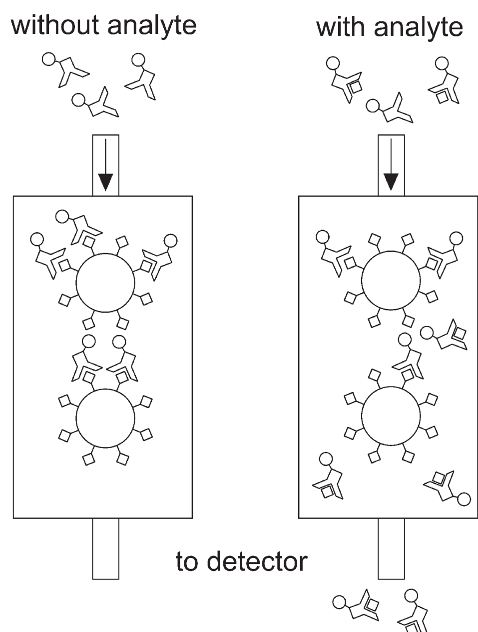

Fig. 7 General principle of non-competitive immunoassays for the determination of haptens (analytes of low molecular mass) using an antigen-column

8 Biomolecules other than antibodies

8.1 Receptor assays

Not only antibodies or antibody fragments are suitable for chromatographic hyphenation. Molecular imprints (MIPs), enzymes, receptors, oligonucleotides and other biomolecules or synthetic analogues can be used for this purpose. Therefore, the concepts which are discussed here should not be considered as limited to antibodies or immunotechniques. Especially in the context of endocrine disruptors (hormone interference), novel assays based on the affinity of human receptors have been presented [59]. As these assays have some toxicological implications, these hyphenated systems can be used to identify compounds with undesirable properties. This is not possible even with highly sophisticated conventional analytical equipment. Recently, a concept was proposed which uses receptors in a receptor-affinity extraction technique analogous to IAE [60]. Subsequently, the extracts are analyzed in an LC-MS-MS device, which may help to identify novel endocrine disruptors. In analogy to indirect ELISAs, a receptor assay (ELRA) has been introduced [61], which might be useful for off-line or on-line coupling to chromatography. The immunodetector mentioned in section 5.1/Fig. 5 was also applied with human estrogen receptors [62], instead of antibodies. In this special case the use of coumestrol as a fluorescent tracer enabled the implementation of a homogeneous detection system without the usual RAM column to separate bound and free label. The mechanism is based on a significant change of the fluorescence properties of the receptor-bound coumestrol compared to the free molecule. A new company [63] has begun to market a system which can perform these assays for high-throughput screening (HTS) tasks.

8.2 Enzyme inhibition assays

Many toxins inhibit specific enzymes. As enzyme tests can often be performed quite easily, for instance, when the purified enzyme is available and chromogenic substrates are known, hyphenation to chromatographic systems has been shown several times. The analysis of highly toxic cyanobacterial peptides (microcystins) could be achieved with an off-line HPLC-PP2A (protein phosphatase 2A) coupling [64]. It also could be shown that the enzyme inhibition is at least 1,000 times more sensitive than the customary UV-detection. Although mostly off-line, the coupling gives valuable information about the toxicity of a sample. It has to be admitted, however, that these simple biochemical tests clearly are not real toxicity tests as they cannot simulate the complexity of the human body. Nevertheless, compounds which are detected as enzyme in-

hibitors have to be considered as potentially dangerous. Regarding the millions of chemicals which have been synthesized up to now, this kind of toxicity screening will have an increasing significance in future – especially considering the reluctance of the public to accept large-scale animal testing.

9 Other separation techniques

9.1 Thin-layer chromatography

Unfortunately, thin-layer chromatography (TLC) today has the reputation of being "low-tech", but one should not disregard its many advantages. For example, the parallelization of many samples (throughput), the possibility of omitting any sample clean-up and finally the low investment and running costs should be taken into account. In addition, after the separation, derivatization reactions are easily carried out with spray reagents. Some publications exist, where an immunological detection step followed the separation on the TLC plate [65]. Some of the methods are similar to blotting techniques.

9.2 Electrophoresis and capillary electrochromatography

Similarly to the topic of antibodies which has to be extended to other binding molecules, the multitude of existing separation techniques should also be taken into consideration. All kinds of electrophoreses (gel electrophoresis, capillary electrophoresis [66], free flow electrophoresis, isoelectric focusing, etc.) could be used as a separation step for the analytes (as in chromatography) and as a separation for bound and free labeled reagents (as in immunoassays) [67]. The most important variant, the immunoblotting technique, will be discussed separately in the next section. Recently, even the on-line hyphenation of immunoaffinity capillary electrochromatography with capillary zone electrophoresis was presented [68]. Interestingly, one of the very first radioimmunoassays (Ekins, 1960 [69]) was based on an electrophoretic separation of bound and free antibody. Later, the simple separation procedures based on precipitation or solid-phase washing steps gained much more acceptance and the electrophoretic techniques were largely forgotten for a long time. Activities in the direction of miniaturization [70] have once more made electrophoretic techniques very attractive.

9.3 Immunoblotting (western blotting)

Many bioanalytical techniques are widely ignored in the classical analytical field. This is especially true in the case of all blotting techniques, which are indispensable tools for the analysis of proteins [71] and nucleic acids. Therefore, although perhaps being the most abundant "immuno-separation" coupling, many analytical chemists are not aware of this fact. The blotting techniques are separated into three main types, southern blotting (introduced by E. M. Southern [72]), which deals with the separation and detection of DNA, northern blotting (RNA) and western blotting (proteins). It should be noted that neither a scientist with the name Northern nor one with the name Western existed in this context and that these names were given by analogy. In the simplest form of a western blot an analyte mixture (proteins) is separated with a gel-electrophoretic technique (e.g., sodium dodecylsulfate polyacrylamide gel electrophoresis, SDS-PAGE). After the separation, the proteins are blotted (transferred) onto a cellulose nitrate ("nitrocellulose") membrane where the proteins are adsorbed. Finally, the cellulose nitrate membrane is "stained" with immunochemical reagents after suitable blocking steps to avoid nonspecific binding. An important advantage of these systems is the high dimensionality, as a two-dimensional electrophoresis (SDS-PAGE combined with isoelectric focusing, IEF) can be coupled with an additional dimension of immunoanalysis, which itself can have several steps. Therefore, it is possible to separate and identify very complex mixtures of proteins (up to 10,000 proteins per gel). Southern blotting in combination with in-situ DNA hybridization, which is a very similar technique, will not be discussed here in detail. However, one has to keep in mind that DNA hybridization is one of the fundamental methods in biotechnology, including all recombinant techniques. In this respect an oligonucleotide is used as selective reagent instead of an antibody. The well-known base-pairing mechanism leads to very specific results when suitable conditions are employed. A special advantage is that oligonucleotides can be synthesized quite easily and even thousands of different sequences can be manufactured in parallel, which could be achieved by a photolithographic technique [73].

9.4 Countercurrent chromatography and field-flow fractionation

Countercurrent chromatography (CCC) can be used very similarly to HPLC, with the important difference that a solid phase is completely lacking. All hyphenations shown for other liquid chromatographic systems might be transferred to CCC, also. Although one might think that a method called affinity countercurrent chromatography [74] might be an immunochromatographic method, the "ligand" in this case is not a biological molecule but a surfactant derivative. Despite the obviously low selectivity and low affinity of the interaction between surfactant and analyte, separations have been quite successful. The many variants of field-flow fractionation (FFF) have not yet received much attention in the immunological field. This may be the reason why corresponding studies seem to be lacking up to now. The separation of polymeric analytes might be achieved quite efficiently with FFF as in this technique separation is achieved essentially according to molecule size.

10 Stationary phases

10.1 Conventional affinity materials

The manufacturing of immunoaffinity cartridges, which could be applied for IAE, can be achieved with a large number of techniques. The most often applied method is the use of activated Sephadex® gels or synthetic polymers, such as Eupergit C®, Toyopearl® or others, which are commercially available. Furthermore, silica supports are widely used, such as amino-, diol- or aldehyde-derivatized silica. The coupling procedures are very straightforward, and successful coupling is achieved quite easily. Sometimes, however, the antibodies are denatured during the immobilization process and only low activities are obtained. In addition, relatively pure antibody preparations are needed. Free amines such as tris buffer (tris(hydroxymethyl)-aminomethane) strongly interfere with many of the coupling chemistries, so that a previous desalting step is needed. In addition, some matrix constituents bind nonspecifically to these polymers, which may lead to low selectivities.

10.2 Sol-gel materials

A very interesting technique for the immobilization of molecules is the sol-gel approach [75], which has also been applied to the immobilization of antibodies [76–78]. The sol-gel technique uses a glass for the entrapment of antibodies. The antibodies are not altered chemically, they are only physically confined. The pores are large enough to enable the largely unimpeded access of the analyte molecules to the antibodies. There is some evidence, also, that the antibodies are stabilized in terms of denaturation and microbial degradation. It has been suspected that the pores are too small for microbial and enzymatic attack. A problem might be the ageing of the material, which changes its properties, for instance the pore-size. One of the most significant advantages of this technique, however, is the low nonspecific binding of the glass matrix. This results in extraordinarily pure extracts in IAE applications. Nevertheless, the preparation and use of sol-gel materials cannot be considered as routine today.

10.3 Molecularly imprinted polymers (MIPs)

Although not an immunological technique, the advent of molecular imprinted polymers (MIPs) [79, 80] should be mentioned. In a paper which pointed the way ahead [81], MIPs were described as synthetic antibodies which might be useful for analytical techniques such as immunoassays. Unfortunately, immunoassay-similar approaches with MIPs require the use of radioactive labels in most cases [82, 83], although first approaches have been shown to overcome this limitation [84]. For the preparation of MIPs one or several monomers are mixed with a relatively large amount of a cross-linker, porogen (solvent) and a radical starter. The desired analyte is dissolved in this mixture. After polymerization the polymer block is ground and extensively extracted with solvents to remove the soluble analyte. This procedure leaves small molecular imprints of the analyte in the polymer which are suitable for the binding of the respective analyte. The above approach is called non-covalent imprinting. Even older is the approach of covalent imprinting, which uses monomer-analyte conjugates, which are subsequently cleaved to achieve a suitable binding site. Rod-like MIPs can be prepared [85] and have been applied to the separation of xanthine derivatives (theophylline and caffeine). The interest in molecular imprinting has risen dramatically in recent years, nevertheless, the approach remains relatively difficult. One of the reasons is the strong nonspecific binding of the polymer itself, which requires optimized loading and washing conditions [86]. In some cases the imprinted polymer shows only a weak improvement in the binding of the analyte in relation to the reference polymer. In addition, the affinities and capacities of MIPs are in most cases much lower than similar antibody columns. In trace analysis the occurrence of analyte-bleeding can be a severe problem as it is very difficult to remove all of the analyte used for imprinting. A big advantage is the robustness of MIPs which can be eluted and cleaned by quite crude methods, without impairing their binding properties. The possibilities for sensor applications which also have some relevance for the construction of immunodetectors are going to be extensively explored [87].

10.4 Cyclodextrins and calixarenes

Even one more step away from antibodies is the use of cyclodextrins and calixarenes. Columns with these affinity molecules are commercially available [88, 89]. The corresponding chromatograms are quite promising. Similarly to MIPs, cyclodextrin columns are mainly used for enantioseparation. Calixarene columns can be considered as reversed phases with unusual selectivity and are used like conventional RP materials in chromatography.

10.5 Restricted access materials (RAMs)

Restricted access materials [90, 91] can be considered to be a combination of a reversed-phase material and an exclusion chromatographic gel. RAMs can be based on silica, which is modified hydrophobically. The outer surface of the particles, however, is hydrophilic. The pore size is selected in such a way that the analytes can enter the pores and therefore are bound to the reversed phase. Polymeric matrix constituents, typically blood proteins, are excluded from the pores and do not stick to the hydrophilic outer surface. The matrix polymers are not retained and exit the column essentially in the void volume. As they cannot enter the pores, almost no surface fouling which could alter the retention characteristics of the material, occurs. RAMs

are mostly used for on-line extraction procedures with very difficult matrices. Generally, the analyte separation is performed on a second column, which is filled with a standard RP material. For immunochromatographic techniques RAMs are useful for the separation of hapten-dye conjugates from their respective antibody complexes. In addition, RAMs have been applied in a more conventional way in an on-line clean-up of difficult samples in hyphenated immunochromatographic systems.

11 Miniaturized techniques

An intense discussion is ongoing on the usefulness of miniaturized analytical techniques [92], which may be considered part of the emerging nanotechnology complex. Quite a long time ago, Widmer [93, 94] began to propagate the μ-TAS (micro total analysis system), which he believed would be the analytical system of the future. Although the beginning of the new millennium may also mark a fundamental reorientation of analytical chemistry, the implementation of miniaturized systems is still in its infancy. The best developed field seems to be capillary electrophoresis (CE), which has some inherent advantages concerning miniaturization. For instance several papers show the application of immunoassays on CE chips [95, 96]. It has to be noted that CE is mostly used for separation of bound and free reagents, not for the electrophoretic separation of similar analytes. Furthermore, capillary electrokinetic chromatography (CEC) may be an even more powerful technique for miniaturization as many proven separation protocols may be translated from HPLC into a CEC method. In a next step the above hyphenations might be transferred to a chip format, obtaining a complete and very efficient "analytical-lab-on-a-chip".

12 Conclusions and outlook

From the material presented, it is clear that increasing attention is being paid to immunochromatographic techniques. In addition, reservations of employing biochemical methods in general seem to be decreasing. Frequently used is immunoaffinity extraction (IAE), which can easily be combined with many other chromatographic, electrophoretic and spectroscopic techniques. As a next step a higher degree of automation for IAE (on-line) seems to be desirable. Other techniques have to be regarded as nonstandard and in most cases only some prototype systems exist. One very interesting approach is the development of advanced immunodetectors or biochemical detectors. This field has much in common with the immunosensor/biosensor area. Perhaps in future these highly selective detectors might be used as frequently in HPLC applications as, for instance, fluorescence detectors today. Miniaturization will remain a major task for the future, also. Many research groups are trying to achieve development of a system, which might be denoted as a true "analytical-lab-on-a-chip" or μ-TAS (micro total analysis system) as supported by Widmer. In these cases, electrophoresis and electrochromatography seem to be of some advantage. For extremely demanding analyses of highest complexity even triple-hyphenations or other sophisticated systems may be necessary. This may be the case if unknown ultratrace compounds have to be identified, quantified and a structural elucidation is unavoidable. Here, for instance, IAE/LC/UV/MS couplings seem to be very useful. To generalize this trend, one might suspect that not only immunochromatographic systems should be used, but that even immunochromatography-spectroscopy hyphenations are needed.

Considering all the systems which have been implemented up to now, it is obvious that the hyphenation of immunological and chromatographic techniques is highly synergistic and beneficial. Thus, in all cases where complex and demanding analytical tasks have to be solved, these combinations will be advantageous. Although much research and development remains to be done, it can be expected that immunochromatographic techniques will gain further significance at the beginning of the 21st century.

Acknowledgments The author would like to thank A. Zeck and D. Knopp, both Institute of Hydrochemistry, Technical University of Munich, for helpful discussions.

References

1. de Frutos M (1995) Trends Anal Chem 14:133–140
2. Irth H, Oosterkamp AJ, Tjaden UR, van der Greef J (1995) Trends Anal Chem 14:355–361
3. Hage DS (1997) J Clin Ligand Assay 20:293–301
4. Ballesteros B, Marco M-P (1998) Food Technol Biotechnol 36:145–155
5. Shahdeo K, Karnes HT (1998) Mikrochim Acta 129:19–27
6. Hage DS (1998) J Chromatogr B 715:3–28
7. Hage DS (1999) Clin Chem 45:593–615
8. Weller MG, Schuetz AJ, Winklmair M, Niessner R (1999) Anal Chim Acta 393:29–44
9. Schuetz AJ, Winklmair M, Weller MG, Niessner R (1999) Fresenius J Anal Chem 363:625–631
10. Winklmair M, Schuetz AJ, Weller MG, Niessner R (1999) Fresenius J Anal Chem 363:731–737
11. Martín-Esteban A, Fernández P, Cámara C (1997) Fresenius J Anal Chem 357:927–933
12. Thurman EM, Mills MS (1998) Solid-phase extraction – principles and practice. John Wiley & Sons, New York
13. Märtlbauer E, Dietrich R, Usleber E (1996) Veterinary drug residues 636:121–131
14. Kondo F, Matsumoto H, Yamada S, Ishikawa N, Ito E, Nagata S, Ueno Y, Suzuki M, Harada K (1996) Chem Res Toxicol 9:1355–1359
15. Coring System Diagnostix GmbH, 64579 Gernsheim, Germany, 1999
16. Dietrich R, Usleber E, Märtlbauer E (1998) Analyst 123: 2749–2754
17. Spark Holland Instrumenten, 7800 AJ Emmen, The Netherlands, 1999
18. Martín-Esteban A, Fernández P, Stevenson D, Cámara C (1997) Analyst 122:1113–1117
19. Cai J, Henion J (1996) Anal Chem 68:72–78
20. Nedved ML, Habibi-Goudarzi S, Ganem B, Henion JD (1996) Anal Chem 68:4228–4236

21. Ohlson S, Zopf D (1993) Weak affinity chromatography. In: Handbook of Affinity Chromatography, Dekker, New York, pp 299–314
22. Ohlson S, Bergström M, Pahlsson P, Lundblad A (1997) J Chromatogr A 758:199–208
23. Giraudi G, Baggiani C (1996) Analyst 121:939–944
24. Remcho VT, Tan JZ (1999) Anal Chem 71:248A–255A
25. Kempe M (1996) Anal Chem 68:1948–1953
26. Kempe M, Mosbach K (1995) J Chromatogr A 694:3–13
27. Kriz D, Berggren Kriz C, Andersson LI, Mosbach K (1994) Anal Chem 66:2636–2639
28. Nagata S, Tsutsumi T, Hasegawa A, Yoshida F, Ueno Y, Watanabe MF (1997) J AOAC Internat 80:408–417
29. Marco M-P, Gee S, Hammock BD (1995) Trends Anal Chem 14:341–350
30. Fishman HA, Greenwald DR, Zare RN (1998) Ann Rev Biophys Biomol Struct 27:165–198
31. Irth H, Oosterkamp AJ, van der Welle W, Tjaden UR, van der Greef J (1993) J Chromatogr 633:65–72
32. Oosterkamp AJ, Irth H, Tjaden UR, van der Greef J (1997) J Chromatogr 787:37–46
33. Miller KJ, Herman AC (1996) Anal Chem 68:3077–3082
34. Oosterkamp AJ, Irth H, Heintz L, Marco-Varga G, Tjaden UR, van der Greef J (1996) Anal Chem 68:4101–4106
35. Önnerfjord P, Eremin SA, Emnéus J, Marko-Varga G (1998) J Chromatogr 800:219–230
36. Lutz ESM, Irth H, Tjaden UR, van der Greef J (1996) J Chromatogr 755:179–187
37. Brecht A, Gauglitz G (1997) Anal Chim Acta 347:219–233
38. Nice E, Lackmann M, Smyth F, Fabri L, Burgess AW (1994) J Chromatogr 660:169–185
39. Tom-Moy M, Doherty TP, Baer LR, Spira-Solomon D (1995) Use of an acoustic wave device as a liquid chromatography detector. In: Biosensor and Chemical Sensor Technology: Process Monitoring and Control, ACS Symposium Series 613. American Chemical Society, Washington, pp 9–18
40. Dickert FL, Hayden O (1999) Fresenius J Anal Chem 364:506–511
41. Mayer M, Muscate A, Bruin GJM, Ehrat M (1998) Chimia 52:313–313
42. Schnappinger P, Schneider E, Märtlbauer E, Terplan G (1996) Food Agric Immunol 8:269–272
43. Morais S, Maquieira A, Puchades R (1999) Anal Chem 71:1905–1909
44. Zeck A, Weller MG, Niessner R (1999) Fresenius J Anal Chem 364:113–120
45. Narang U, Gauger PR, Ligler FS (1997) Anal Chem 69:2779–2785
46. Zuk RF, Ginsberg VK, Houts T, Rabble J, Merrick H, Ullman EF, Fischer MM, Sizto CC, Stiso SN, Litman DJ (1985) Clin Chem 31:1144–1150
47. Durst RA, Siebert STA, Reeves SG (1993) Biosens Bioelectron 8:xiii–xv
48. de la Torre R, Domingo Salvany A, Badia R, Gonzalez G, Mc Farlane D, San L, Torrens M (1996) Clin Chem 42:1433–1438
49. Puchades R, Maquieira A, Atienza J, Montoya A (1992) Crit Rev Anal Chem 23:301–321
50. Fintschenko Y, Wilson GS (1998) Mikrochim Acta 129:7–18
51. González-Martínez MA, Morais S, Puchades R, Maquieira A, Abad A, Montoya A (1997) Anal Chim Acta 347:199–205
52. Dietrich M, Krämer PM (1995) Food Agric Immunol 7:203–220
53. Krämer PM, Baumann BA, Stoks PG (1997) Anal Chim Acta 347:187–198
54. Meyer UJ, Trau D, Key G, Meusel M, Spener F (1999) Biocatal Biotransform 17:103–124
55. Turiel E, Fernández P, Pérez-Conde C, Gutiérrez AM, Cámara C (1998) Talanta 47:1255–1261
56. Weller MG, Zeck A, Pfortner P, Simon E, Niessner R (1999) Intern J Environ Anal Chem 75 : 201–215
57. Freytag JW, Dickinson JC, Tseng SY (1984) Clin Chem 30:417–420
58. Bauer CG, Eremenko AV, Kühn A, Kürzinger K, Makower A, Scheller FW (1998) Anal Chem 70:4624–4630
59. Oosterkamp AJ, Hock B, Seifert M, Irth H (1999) Trends Anal Chem 16:544–553
60. Seifert M, Brenner-Weiß G, Haindl S, Nusser M, Obst U, Hock B (1999) Fresenius J Anal Chem 363:767–770
61. Seifert M, Haindl S, Hock B (1999) Anal Chim Acta 386:191–199
62. Oosterkamp AJ, Villaverde Herraiz MT, Irth H, Tjaden UR, van der Greef J (1996) Anal Chem 68:1201–1206
63. Screentec, 2333 CA Leiden, The Netherlands, 1999
64. Sherlock IR, James KJ, Caudwell FB, MacKintosh C (1997) Natural Toxins 5:247–254
65. Pestka JJ (1991) J Immunol Meth 136:177–183
66. Bao JJ (1997) J Chromatogr B 699:463–480
67. Tao L, Kennedy RT (1996) Anal Chem 68:3899–3906
68. Thomas DH, Rakestraw DJ, Schoeniger JS, Lopez-Avila V, Van Emon J (1999) Electrophoresis 20:57–66
69. Ekins RP (1960) Clin Chim Acta 5:453–459
70. von Heeren F, Verpoorte E, Manz A, Thormann W (1996) Anal Chem 68:2044–2053
71. Dunbar SD (1994) Protein blotting: A Practical Approach, Oxford Univ Press, Oxford
72. Southern EM (1975) J Mol Biol 98:503–517
73. Chee M, Yang R, Hubbell E, Berno A, Huang XC, Stern D, Winkler J, Lockhart DJ, Morris MS, Fodor SA (1996) Science 274:610–614
74. Ma Y, Ito Y (1996) Anal Chem 68:1207–1211
75. Dave BC, Dunn B, Selverstone Valentine J, Zink JI (1994) Anal Chem 66:1120A–1127A
76. Zühlke J, Knopp D, Niessner R (1995) Fresenius J Anal Chem 352:654–659
77. Cichna M, Knopp D, Niessner R (1997) Anal Chim Acta 339:241–250
78. Bronshtein A, Aharonson N, Avnir D, Turniansky A, Altstein M (1997) Chem Mater 9:2632–2639
79. Wulff G (1995) Angew Chem Int Ed 34:1812–1832
80. Shea KJ (1994) Trends Polymer Sci 2:166–173
81. Vlatakis G, Andersson LI, Müller R, Mosbach K (1993) Nature 361:645–647
82. Ramström O, Ye L, Mosbach K (1996) Chemistry & Biology 3:471–477
83. Andersson LI (1996) Anal Chem 68:111–117
84. Piletsky SA, Terpetschnig E, Andersson HS, Nicholls IA, Wolfbeis OS (1999) Fresenius J Anal Chem 364:512–516
85. Matsui J, Miyoshi Y, Matsui R, Takeuchi T (1995) Anal Sci 11:1017–1019
86. Mayes AG, Mosbach K (1997) Trends Anal Chem 16:321–332
87. Kriz D, Ramström O, Mosbach K (1997) Anal Chem 69:345A–349A
88. Chiralsep, 76170 La Frenaye, France, 1999
89. Synaptec, 17489 Greifswald, Germany, 1999
90. Boos KS, Rudolphi A (1997) LC-GC 15:602–611
91. Rudolphi A, Boos KS (1997) LC-GC 15:814–823
92. Kricka LJ (1998) Clin Chem 44:2008–2014
93. Widmer HM, Erard J-F, Grass G (1984) Intern J Environ Anal Chem 18:1–10
94. Manz A, Graber N, Widmer HM (1990) Sensors and Actuators B1:244–248
95. Schmalzing D, Koutny LB, Taylor TA, Nashabeh W, Fuchs M (1997) J Chromatogr 697:175–180
96. Chiem N, Harrison DJ (1997) Anal Chem 69:373–378