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Review

Manufacture of recombinant proteins with safe and validated chromatographic sorbents

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Abstract

Purification of recombinant proteins to achieve homogeneity, purity, consistency and potency as required for therapeutic proteins and in vivo diagnostics is performed under stringent and validated conditions. As liquid chromatography is one of the major technologies used for this purpose, it has to be carried out according to special regulatory guidelines. One of the reported aspects is the long-term consistency of a chromatographic process and validation of its operation; other aspects described are more sorbent orientated. In-place cleaning and sterilization are also very important aspects, the efficiency of which is dependent on the chosen working conditions and the chemical nature of the sorbents. Drastic cleaning may deteriorate the chromatographic matrices, releasing chemicals that may contaminate the biologicals of interest, while modifying the behaviour of the chromatographic columns. Moreover, leachable compounds, when present, could have adverse effects in case of high toxicity. Determination of leaching levels and toxicity tests are part of the validation steps to turn chromatographic separations into consistent, effective and safe production processes for biologicals.

Contents

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1. The context of recombinant protein purification

About one decade after the introduction of the first recombinant DNA-derived protein, recombinant DNA technology is now broadly accepted for the production of therapeutic proteins, vaccines, diagnostics, fine chemicals and food additives and also enzymes used for processing alcoholic and non-alcoholic beverages [1]. In the early days of rDNA technology, the uncertainty about how impurities and DNA contamination, when intravenously injected, could affect an organism led to the establishment of very stringent regulations, in spite of the much higher quality of the rDNA products compared with conventional biologicals. One of the first recommendations was published by WHO in 1983 and was based on a report of a consultation held in Geneva in 1983 [2]. According to the US Code of Federal Regulations (CFR21), a biological product could be any virus, therapeutic serum, toxin, antitoxin or analogous product applicable to the prevention, treatment or cure of diseases or injuries of man; this definition includes both conventional and recombinant DNA products. Early regulations for rDNA derived products, published by the Center of Biologics Evaluation and Research (CBER), such as "*Points to Consider in the Production and Testing of New Drugs and Biologicals Produced by Recombinant DNA Technology (1985)*" or "*Points to Consider in the Characterization of Cell Lines to Produce Biologicals (1987)*", demonstrate the high concern to define quality standards. In this concept, products had to be purified to homogeneity as defined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing (IEF), amino acid analysis, absence of DNA (<10 pg per dose) and pyro-

genic substances, absence of host cell contaminants and contaminants from culture medium. EEC legislation followed the same philosophy as WHO and CBER. A special EEC Biotechnology/Pharmacy working party was set up in 1985 [3] with two main tasks: (1) to advise the Committee for Proprietary Medicinal Products (CPMP) on individual applications for marketing authorization of medicinal products derived from biotechnology, and (2) to establish specific guidelines on the quality and safety of biotechnological products.

The five following guidelines were prepared by the working party in accordance with competent authorities and industry and published by the Office for Official Publications, catalogue number CB-55-89-843-EN-C:

- Production and quality control of monoclonal antibodies of murine origin (June 1987).
- Production and quality control of medicinal products derived by recombinant DNA technology (June 1987).
- Preclinical safety testing of biotech medicines (June 1988).
- Production and quality control of cytokines (February 1990).
- Production and quality control of human monoclonal antibodies (July 1990).

A review on the regulatory considerations for rDNA products was recently published by Kozak et al. [4].

The frequent iatrogenic transmissions of infectious diseases such as AIDS, hepatitis, cytomegaly and others by therapeutic proteins derived from human blood has led to reconsideration of the quality demands for conventional biologicals [5].

The basic principles of how high-quality standards can be achieved by means of rDNA technology were then defined as follows:

- Use of a defined host cell line or transgenic animal.
- Sequence of purification steps which provide sufficient clearance of adventitious agents.
- Careful validation of all unit operations.
- Use of defined culture media.

At present, the high purity requirements and the high numbers of log-steps in impurity clearance, can only be met when column chromatography is used in the recovery and purification of rDNA products [6]. That is the reason why liquid chromatography plays such an important role in rDNA technology, and why it is frequently used in the manufacture and isolation of conventional biologicals such as serum proteins. Column chromatography is actually the sole technique that provides high recovery, high resolution and high throughput (except for size exclusion), and which can be scaled up to process several tons of product per annum.

2. Potential impact of solid sorbents on proteins

Packed-column chromatography is a method used for protein production, when high resolution and high selectivity are necessary. Compounds such as pyrogens, DNA, virus particles and proteins are macromolecules with amphoteric properties. Owing to these properties, proteins can be separated from other compounds, the amphoteric nature of proteins being more pronounced. The selectivity of ion-exchange chromatography and hydroxyapatite chromatography for proteins is based on this property. Proteins also have hydrophobic patches on their surfaces. Hydrophobic interaction chromatography (HIC) and reversed-phase chromatography (RPC) use these characteristics of a protein. Biospecific interaction exploiting the biological interaction between a protein and its natural ligand is used in affinity chromatography. Biomimetic interactions exploit the mimicking of a ligand–protein interaction, e.g., triazine dyes mimic substrates for oxidoreductases [7] and tentacle cation exchangers mimic DNA [8]. Metal chelate affinity chromatography [9] utilizes the coordination of immobilized divalent metal ions with accessible histidine residues at the surface of a protein [10]. Both biomimetic chromatography and metal chelate chromatography are also named pseudo-affinity chromatography. The size of a molecule is another characteristic used in size-exclusion chromatography, which is an essential unit operation in manufacturing therapeutic proteins. Both removal of oligomers

and desalting can be carried out very efficiently by this technique. Besides the specific interaction of a protein with a resin, unwanted interactions, called non-specific interaction, may also take place. This non-specific binding (expressed as free energy of the reaction under equilibrium conditions) is often much stronger than the specific interactions. These binding forces may cause denaturation of proteins and in special cases "irreversible" binding may also occur. Non-specific binding phenomena are often responsible for loss of yield in protein chromatography and most frequently the nature of the sorbent plays a dominant role.

Cross-linking reactions are frequently applied to improve the hydraulic stability of inert agaroses and dextran. The linker introduces hydrophobic sites or even charged groups [11] into the gel. On the other hand, silica gels and rigid organic polymers such as polystyrene may be coated with inert polymers to cancel non-specific binding and to create sorbents that can be used for very large-scale production. However, coatings may not be complete and consequently Si–OH groups or hydrophobic patches are left in the sorbent, which may lead to non-specific interactions with proteins. A 100% inert sorbent is in fact still a dream, or cannot be produced in practice. At present five types of sorbents are marketed, categorized according to the physical nature of penetration of a protein into the matrix.

From Table 1, one can infer that different types of materials are available for protein purification. The type of penetration and also the adsorption–desorption mechanisms influence the

Table 1
Comparison of sorbents for protein chromatography, described according to the physical nature of penetration into the sorbent particle

Mode of protein penetration into sorbent particle	Feature of pore structure or shape of particle	Time required for binding	Dynamic capacity	Example of sorbent	Ref.
Conventional diffusion into pores	Rigid pore network restricts movement of protein	Moderate	Moderate	Coated silica (Spherodex)	99
				Dextran (Sephacryl)	100
				Agarose (Sepharose)	101
				Polyacrylamide (Bio-Gel)	102
Convection into the sorbent particles (perfusion)	Highly porous network with trajecting pores and diffusive pores	Extremely short	Extremely low	Polystyrene (Poros)	103
Diffusion into a viscoelastic fluid	Only diffusive pores	Short	High	Tentacle (Fractogels)	54
				Gel-filled pores (HyperD)	104
No penetration	Non-porous	Extremely short	Extremely low	–[a]	
Diffusion and minimal convection	Continuous solid phases, monoliths	Unknown	Unknown	–[b]	

[a] No application for industrial-scale chromatography.
[b] Not yet on the market; membranes are classified in this category.

clearance of adventitious agents. Reviewing the modes of interaction between the protein in solution and the solid supports would exceed the scope of this paper; however, it is possible to classify these interactions into three main categories, ionic interactions, hydrophobic interactions and bioaffinity binding based on complex multivalent molecular recognition. Examples of affinity interactions are:

- enzyme–substrate;
- enzyme–inhibitor;
- enzyme–substrate analogue;
- antibody–antigen;
- DNA binding protein–oligonucleotide;
- protein mimicry of natural counterpart.

Very complex affinity chromatography may be required for protein purification, when high purity is required together with high recovery. With such an approach, high purity can be obtained in a single step and the protein can be extensively washed, whilst bound to the column [12].

3. Adventitious agents in the production of recombinant proteins

Production of a recombinant protein is not a "clean" process like the chemical synthesis of a macromolecule with a limited amount of by-products. Many adventitious agents are present in a crude bacterial extract or in a cell culture supernatant and must be selectively removed. Most frequently the biological is present as a minor component when compared with the amount of foreign material and moreover it is also very diluted. The main constituents are shown in Fig. 1.

In addition to the expressed protein, host cell proteins, media proteins, host cell DNA/RNA, media DNA/RNA, cell wall components, salts, lipids, and other small-molecule metabolites are present. In the case of yeast extracts, serum or colostrum [13] is not added to the basal culture medium and contamination from the culture medium can be neglected. Using animal host cells, possible infection by viruses must also be considered (e.g., murine incomplete retroviral particles).

Removal of all the mentioned agents is the goal of chromatography. Clearance of these agents has to be carefully validated, because in the sense of the definition of a biological, the rDNA-derived protein is not only described by its chemical structure, but is also characterized by an operational definition, in the sense that the way in which the protein is produced is part of the description of the substance. This conservative approach, which guarantees consistency of a product over the years, sometimes may be responsible for hindrance of technological progress, which may also lead to a cost reduction.

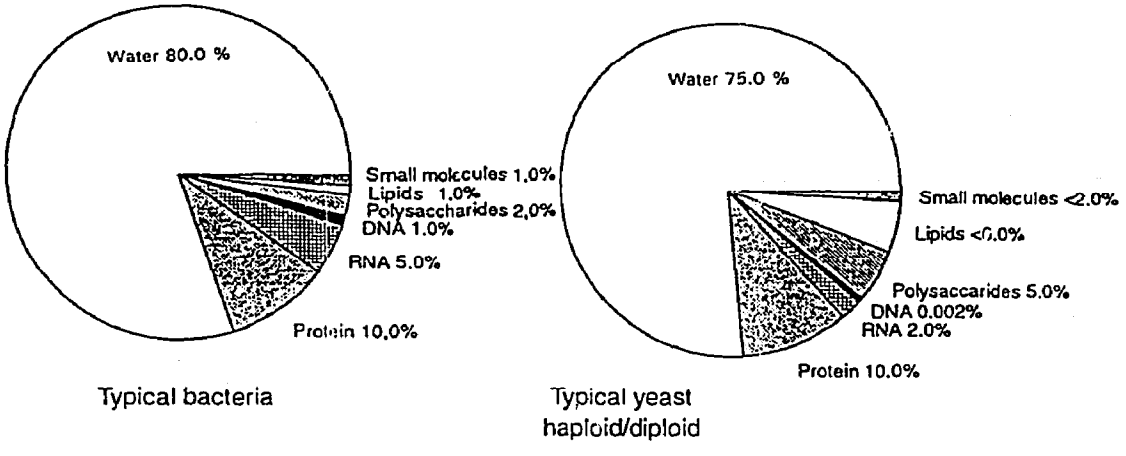

Fig. 1. Main components of *E. coli* and yeast cells.

3.1. Host and media proteins

In addition to water, proteins are the major adventitious agents of an rDNA protein in a crude supernatant, cell homogenate, whey, blood, etc.

When the protein is deposited in inclusion bodies, the removal of the bulk of proteins can be simply achieved by repeated washings of the inclusion bodies. When the protein is soluble or associated with membranes, column chromatography is the method of choice, even in the very early steps. Although fluidized beds solve particular technological problems occurring with particles in suspension and thereby allow faster operation, the clearance of adventitious agents may be lower [14] than with packed beds.

3.1.1. Formation of heterodimers

A critical problem in purification is the formation and separation of heterodimers. When a dimer is expressed in a host cell that also produces a similar protein with a similar three-dimensional structure as well as similar specificity and behaviour, additional efforts must be made to achieve complete isolation. An excellent example is the production of recombinant proteins in transgenic animals. Human haemoglobin overexpressed in transgenic swine had a highest level of expression of 24% human (32 g/l) and 30% human α/pig β hybrid (40 g/l) haemoglobin in one transgenic pig [15].

3.1.2. Detection of protein contaminants

A powerful method for the detection of contaminant proteins is polyacrylamide gel electrophoresis under reducing and non-reducing conditions in combination with silver staining and immunological detection (Western blots). Antisera against media proteins or antisera made against dummy preparations are useful tools. Protein determination by conventional techniques using dyes, fluorescent dyes or spectrophotometry at 214 or 280 nm is not sensitive enough to detect traces of protein impurities.

3.2. Protein-degrading enzymes

Proteases are frequently present in cell homogenates. They originate from regular cell lysis or may be secreted by a cell line, and must be efficiently eliminated. Inhibition of the protease activity and fast removal in the initial purification steps are then essential. Less attention has been paid to glycosidases, which may also inactivate or modify proteins. An example is the exoglycosidase activity of insect host cells, such as *Spodoptera frugiperda*, *Trichopulsia ni*, *Bombyx mori* or *Malacosoma disstria*. All four insect cell lines contain N-acetyl-β-glucosaminidase, N-acetyl-β-galactosaminidase, β-galactosidase and sialidase activities [16]. Proteases can be easily detected by an assay using agarose plates, where a protein is added to the melted agarose before formation of the gel plate. The assay is not very sensitive, but gives a quick overview of what happens during a purification process. If a sensitive assay is required, the only way to detect proteases is by protein radiolabelling. After acid precipitation, the supernatant is harvested and counted in a scintillation counter. Partially degraded proteins and completely degraded proteins will not precipitate [17].

3.3. DNA and RNA

DNA and RNA are considered as possible hazardous compounds. Iatrogen diseases caused by DNA or RNA impurities from an rDNA therapeutic have not been reported previously, to our knowledge. The purity requirements for DNA and RNA are still the same as at the beginning of the rDNA era, because DNA and RNA also originate from media additives such as yeast extracts and serum components. A low DNA and RNA content is also an indication of excellent process design and performance. The use of bovine serum as a medium supplement increases the risk of viral contamination, which is more likely than contamination with substantial amounts of DNA and RNA.

A number of methods are available to detect traces of DNA in protein solutions. Immunologi-

cal assays, such as enzyme-linked immunosorbent assay (ELISA) [18], are very helpful for the detection of high DNA concentrations. This method is useful for in-process control in the initial purification steps. The handling is less critical and the assays are very rapid when compared with more sensitive assays. For higher sensitivity, two methods are applicable to detect small amounts of DNA and RNA, the hybridization assay [19] and the enzyme immunoassay using two high-affinity DNA-binding proteins conjugated with a linker molecule, e.g., biotin, for specific capture of the DNA complex on a membrane anti-DNA monoclonal antibodies conjugated to urease for signal generation; the signal is amplified by a silicon sensor-based system [20]. The assay can detect 2 pg of DNA with a quantification coefficient of variation of less than 10% in the range 10–200 pg.

Quantitative PCR is likely to be used increasingly in the future for the detection of traces of DNA. The method is very selective, although this is not necessary for the purpose of monitoring DNA clearance during protein purification. A characteristic DNA sequence, which is expressed as a stable fraction of the whole DNA, can be used to measure the overall DNA content [21]. Moreover, PCR could also be used for the detection of viruses.

A method for validating DNA/RNA removal is by spiking experiments using radiolabelled DNA/RNA. The DNA can be labelled in vitro by nick translation using ^{32}P- or ^{35}S-labelled nucleotides or in vivo by feeding the cells with radiolabelled nucleotides [22].

3.4. Pyrogens

Pyrogens are cell wall components of Gram negative bacteria having a common core structure [23] (Fig. 2), but no defined size and uniform chemical structure. They induce febrile reactions in mammals [24]. Their molecular mass varies from less than 10 000 to 300 000.

The classical detection method is the rabbit test, in which a certain amount of sample is injected into the animals and the body temperature is measured over a defined time period with an anal thermometer. If a certain temperature sum in several rabbits is not exceeded, the substance is considered as apyrogenic. The method detects a broad range of fever-inducing substances, but the sensitivity is low.

The limulus amoebocytes lysate assay (LAL)

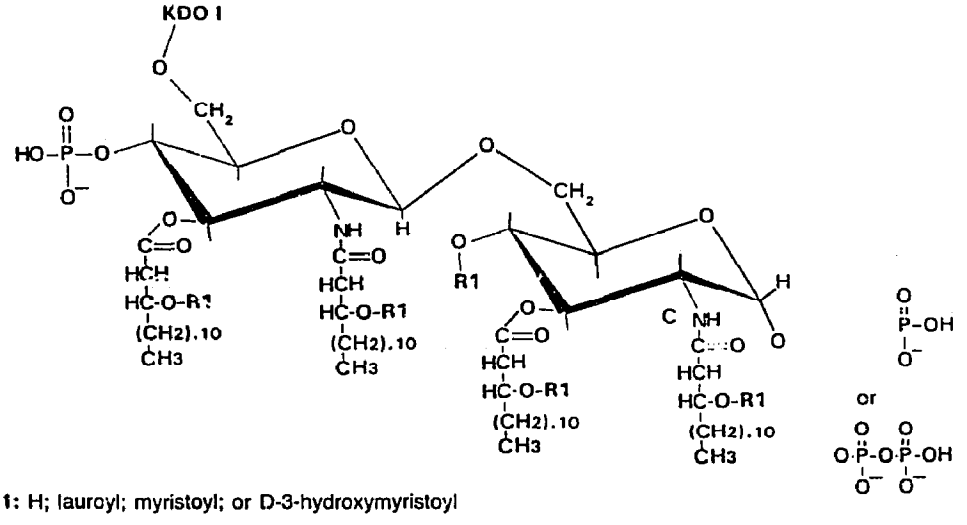

Fig. 2. Core structure of bacterial endotoxins, lipid A; KDO is 3-deoxy-D-mannooctulsonate. The pyrogenic part of the endotoxin is the lipid A core, which is very conserved among all bacterial strains.

is another pyrogen detection method [25] based on the activation of an enzyme cascade extracted from the horseshoe crab (*Limulus polyphemus*). The lysate tends to form a gel when endotoxins from Gram negative bacteria are present. Three main types of assays involving LAL are in use:
- Coagulation assay (semiquantitative);
- turbidimetric assay (semiquantitative);
- chromogenic assay (quantitative).

The sensitivity of these assays is in around 0.01 EU/ml [12 EU (endotoxin units) correspond to about 1 ng].

A pyrogen-free solution does not necessarily have to be sterile, the LAL assay does not detect intact living microorganisms at low germ counts and cell wall components from Gram positive bacteria are also not detected.

3.5. *Viruses, bacteria and transmissible degenerative encephalopathies (TDE)*

Viruses, mycoplasma and bacteria are possible contaminating agents from starting solutions originating from the host cell or from the environment. Mycoplasma contamination of a partially purified product caused by the environment is unlikely to occur, because the organism requires very complex growth conditions. Mycoplasma are parasitic microorganisms that depend on the metabolism of host cells.

Bovine spongioform encephatopathy (BSE) is caused by another agent, which is suspected to be present in bovine material (such as calf sera or bovine trypsin, which is used in the propagation of anchorage-dependent cells). While the suspicion of transmission from the aforementioned sources is not completely disproved, the serum and animal-derived additives should be carefully selected and whenever possible avoided. Only material from BSE-free countries can ensure the absence of this adventitious agent.

BSE [26] is a new member of the transmissible degenerative encephalopathies (TDE) which include scrapie of sheep and Creutzfeld–Jacob disease (CJD) in man. The causative agents are not well characterized but share many unusual properties, including a relative resistance to classical inactivation methods. As a consequence, one must always take into account that a completely validated process for virus inactivation using physical and chemical methods (heat, extreme pH) may not affect TDE. Therefore, chromatographic methods may become very useful for providing more safety concerning TDE clearance. There is good chance of removing these particles selectively.

3.5.1. *Detection methods for bacteria and fungi*

Plate count and MPN (most probable number) tests are the best known methods for counting bacteria. Depending on the growth media and conditions used, a very high selectivity can be achieved. To recover partially destroyed bacteria, the cells should be grown at low temperature. A test system described by Jungbauer and Lettner [27] is a modification of sterility testing of bioreactors. The packed column is filled with caso bouillon after sanitization and incubated. No growth after a certain period is proof of sterility.

As model microorganisms for challenging a packed column, the following species are commonly used: *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeroguinosa*, *Candida albicans* and *Archeoplasma ladwadii*. *Aspergillus niger* spores and *Bacillus subtilis* spores, are recommended model organisms for validation studies because of their particular resistance to chemicals.

3.5.2. *Detection methods for viruses*

Cell culture assays, PCR, in vivo inoculation into small animals and transmission electron microscopy are the methods commonly used. Inoculation into eggs is not used for this purpose. Recently, a highly specific PCR assay was developed to detect the presence of the ecotropic, xenotropic and mink cell focus-forming classes of murine leukaemia viruses (MuLVs) in samples derived from cultured cells and cell free supernatants [28]. The assay can be used to detect and determine any viral contaminant in cell culture supernatant, ascites fluid, process validation samples and final products.

The probability of detecting small numbers of virus particles should always be taken into con-

sideration [29]. At low virus concentrations (e.g. in the range 10–1000 infectious particles per litre), it is evident that a sample of a few millilitres may or may not contain infectious particles. The probability P that a sample does not contain infectious viruses is described by the following equation:

$$P = \left(\frac{V - v}{V}\right)^n \quad (1)$$

where V is the overall volume of the material to be tested, v the volume of the sample and n the absolute number of infectious particles statistically distributed in V. When V becomes $V \gg v$, the probability of a false-negative (p) result follows the Poisson distribution and is expressed by

$$p = e^{-cv} \quad (2)$$

where c is the concentration of the virus. For statistical reasons the sample should always be drawn where the highest titre is expected.

3.5.3. Detection methods for TDE

The bioassay in animals is the only system available for detecting and measuring residual infectivity; various animal species and genotypes have been used, which complicates the problem of comparing data. Western blot analysis is available for BSE, but the sensitivity may be too low.

4. Validation of separation conditions

The validation of separation conditions is the only way to have the highest level of probability for a consistent separation process. At the beginning, the separation efficiency in preparative or large-scale chromatography is mainly affected by the packing quality, the ligand density and the composition of the mobile phase. During successive cycles partial blockage of the interaction sites diminishes the sorption capacity and may lead to sample blockage. Drifts in sorption characteristics of a protein to the sorbent can happen and as a consequence changes of the peak shape and positioning occur. This may result in a decrease in yield and purity. To avoid both problems (changes in shape and position), special separation methodologies must be defined, validated and executed.

4.1. Validation of packing

The packing is simply checked by determining the number of theoretical plates (N) of the column defined by

$$N = 16\left(\frac{t_r}{w}\right)^2 = \left(\frac{M_1}{\bar{M}_2}\right)^2 \quad (3)$$

where t_r is the retention time of a substance and w is the peak width at the base of the peak determined by the tangential method; w corresponds to 4σ (standard deviation of a Gaussian-shaped peak). M_1 and $\bar{M}_2$ are the first peak moment and second central peak moment, respectively. Functionally, a chromatographic peak can be simply considered as a time (volume) distribution of the chromatographic height $h(t)$ at any retention time (volume) t. The statistical moments of the peak are mathematically defined as

$$M_0 = \int_0^\infty h(t)\,dt \quad (4)$$

the zeroth order moment and

$$M_n = \int_0^\infty t^n h(t)\,dt / M_0 \quad (5)$$

the nth moment [30].

To meet the practical conditions, the moments cannot be calculated by integration from the start time until infinity. Therefore, a decision criterion has to be introduced when the calculation is stopped. The integral obtained by this method is called the virtual moment M'. The first and nth virtual moments are calculated by following equations:

$$M'_0 = \sum_0^n \left[(t_n - t_{n-1}) \cdot \frac{R_n + R_{n-1}}{2}\right] \quad (6)$$

$$M'_n = \sum_0^n \frac{t_n \cdot (t_n - t_{n-1})(R_n + R_{n-1})/2}{M'_0} \quad (7)$$

R is the response in μV and has to be corrected

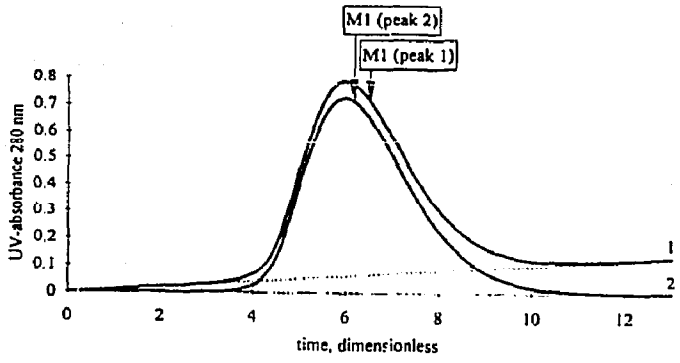

Fig. 3. Calculation of peak moments of peaks with and without baseline drift.

in the case of baseline drift. It has to be verified that R is the linear detection range.

When peak moments are used for the calculation of theoretical plates, it is important to take into account that the moment calculation is very sensitive to tailing and baseline drifts. For instance, peaks with identical retention times of the peak maxima and identical areas, just differing in the baseline, show different moments (Fig. 3).

Graphical methods to determine the packing quality are much more rugged. Calculation of asymmetry (A), also called the tailing factor, by simply dividing the peak width at half-height by that at 10% height:

$$A = \frac{a + b}{2a} \tag{8}$$

indicates clearly changes in the packing and also in the overall set-up (for explanation, see Fig. 4).

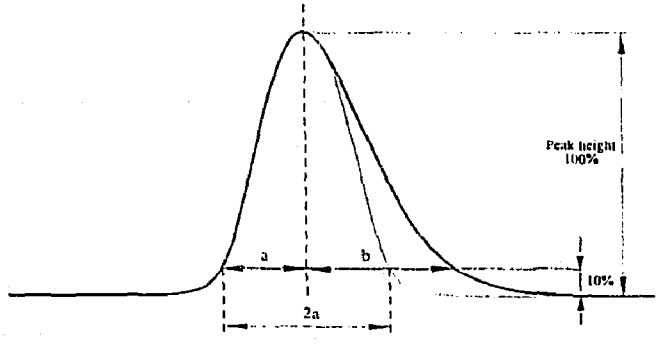

Fig. 4. Calculation of the tailing factor of a peak at 10% of peak maximum.

Packing is validated by pulse experiments. The available substances applicable for these experiments are very limited. NaCl is a very useful substance for determining N. Sodium chloride is widely used and non-toxic, and it is a component that is present in almost all protein purification processes. Validation of the removal of traces of sodium chloride in the column after determination of N is not necessary.

4.2. Validation of ligand density

Validation of the ligand density (ion-exchange groups, hydrophobic chains or affinity ligands) cannot be accomplished by such simple pulse injection of salt solutions or other small substances. Ligand availability loss during the lifetime of a column has to be checked by a small-scale experiment and a fixed cycle number is defined. Theoretically it is possible also to use the peak parameters as decision criterion for ligand density. Loss of ligand availability causes a shorter elution time but broader elution as can be immediately seen from Eq. 9. According to Kucera [31], the first peak moment M_1 gives the retention time, and M_1 can be expressed as

$$M_1 = \frac{\int_0^\infty c(L,t)t\,dt}{\int_0^\infty c(L,t)\,dt} = \frac{L}{u_0}[\varepsilon_T + (1-\varepsilon)\rho_p K_L Q_{max}] + t_0/2 \tag{9}$$

Assuming linear adsorption conditions, Q_{max} (maximum capacity as a function of ligand density) is directly proportional to the retention time; L is the column length, ε is the void fraction, u_0 is the linear velocity, ρ_p is the particle diameter and K_L is the equilibrium constant. This simple relationship shows that the retention time t is dependent on Q_{max}. Further mathematical treatment for the higher moments has been published by Arnold et al. [32].

Halperrin et al. [33] studied the effect of ligand densities on peak broadening in detail using a cellular protein extract and HIC columns.

Partial or progressive fouling, partial ligand

inactivation or ligand leakage may be responsible for ligand leakage availability. Leakage is a complex phenomenon and a detailed description is given in the following sections.

4.3. Validation of mobile phase composition

The composition of the mobile phase is usually validated by an exact protocol for weighing in the particular substances and the operational definition of how the buffer is prepared. More care has to be taken when the buffer is prepared by dilution from a stock dilution. Shifts of pH may occur and often additives such as amino acids may be in a concentration range that is close to the solubility limit. CO_2 from air can also cause problems if an inappropriate buffer is used or if an insufficient molarity is chosen.

The correct buffer composition can be adequately checked by measuring three different values: pH, osmolarity and conductivity. If all three values are within the predefined range, the identity of the substances and the correct preparation are confirmed. Validation of the analytical methods and the equipment is well known and has been described in numerous publications.

4.3.1. Osmolarity

There is a close relationship between the concentration of the solute and the freezing point. The cryoscopic constant K_c is a property of a solvent and can be measured as the lowering of the freezing point (ΔT); for water $K_c = 1.86$. By knowing the cryoscopic constant, the number of dissolved ions or molecules, n, can be measured:

$$\Delta T = K_c n \tag{10}$$

4.3.2. Conductivity

Conductivity may also be used both as a standard method to determine if the molarity of a given buffer is correct and for on-line monitoring. The molar ion conductivity or equivalent conductivity (Λ_i) of an electrolyte is a property of a substance and is defined as

$$\Lambda_i = \frac{\chi}{C_i} \tag{11}$$

where χ is the electrical conductivity and C_i is the concentration of the ion. Further, the molar conductivity is defined as

$$\Lambda = \frac{\chi}{C} \tag{12}$$

where C is the concentration of the electrolyte. An empirical relationship between the concentration of the electrolyte and the molar conductivity exists and can be easily utilized for validation:

$$\Lambda = \Lambda^0 - \sqrt{C} \tag{13}$$

where Λ^0 is the limiting conductivity, defined by $\Lambda^0 = \lim_{C \to 0} \Lambda$. This relationship allows the accurate measurement of the salt concentration of diluted buffers by conductivity. Conductivity is highly temperature dependent, which implies that measurements should be made at constant temperature or temperature corrections should be applied.

pH and conductivity or pH and osmolarity characterize salt buffers sufficiently. The identity of the ingredients obviously has to be checked by other analytical methods.

4.4. Validation of separation conditions

Validation of separation conditions may also be carried out on small-scale of scaled-down production columns. Variations of ligand density, deposition of material in long-term production, etc., are detected by resolution as a simple measure of chromatographic performance. The resolution (R_s) of two standard proteins is also often used for judging the separation efficiency after studying the lifetime or cycle time of a column.

The cycle time is not an adequate parameter to express the robustness of a sorbent. Often the contact time (e.g., how long a sorbent can withstand particular conditions) gives an indication of the lifetime. The cycle time is determined as the functional stability of a scaled-down column using several proteins for the control of separation. Standard proteins that are very stable should be used, whenever it is possible to use the product of interest also in the functional stability assay [34]. The use of immunoglobulin

G (IgG) as a standard protein should be avoided, because it is not a defined protein concerning isoelectric point, exact size and hydrophobicity. Antibodies consist of hypervariable regions, variable regions and constant regions. The isoelectric point is a characteristic of an immunoglobulin in addition to the composition. For comparative reasons, a monoclonal antibody or cheaper proteins such as cytochrome *c*, ribonuclease, chymotrypsinogen, bovine serum albumin (high-purity grade) or other proteins which are very homogeneous (no isoenzymes or isoproteins, no length and charge variants) should be used.

A critical parameter in protein purification is tailing. Tailing causes losses, diluted fractions and increases in process time. Tailing often increases with increasing cycle number and is a clear indication of ageing. A perfectly validated process should take this phenomenon into account. Decision criteria have to be defined concerning the extent of tailing accepted. If the protein is eluted in a fairly pure form, a simple method for determining the tailing factor *A* can be applied. When a protein is eluted together with other contaminants, the tailing factor cannot be derived from the chromatogram. Depending on the process, fractions are collected and purity criteria are used for pooling fractions, then the actual peak volume can be used as a measure of tailing. In processes where the purity ratio of each step or in a single step is very critical, these criteria have to be carefully elaborated. When the size of a column is changed very often, the external peak broadening effects change [35]. Fortunately, in most instances, the peak broadening decreases with the scale. That means that the situation is improved. For very large scales, again the extra-column band broadening effects increase.

4.5. Column regeneration

The adsorption–desorption process of a protein on a column is not a completely reversible process. Therefore, the cleaning or regeneration is an important step, which may prolong the lifetime of a sorbent when it is correctly performed. The hysteresis between adsorption and desorption, which is observed in nearly all sorption processes, necessitates more careful regeneration.

Several low-volume–high-value processes circumvent the problem of regeneration only by discarding the sorbent after a single use. However, in most instances, to satisfy economic requirements, a sorbent must sustain a number of cycles. The initial purification steps are more exposed to irreversible deposition of contaminants on the sorbent surface than sorbents used for advanced purification steps or polishing. Many impurities adhere to the sorbent, because they have a higher distribution coefficient under the applied conditions. Some of them adhere so tightly on the sorbent surface that it is almost impossible to remove them without destroying the sorbent.

In this context, immunoaffinity columns and other sorbents with immobilized proteins or sensitive ligands are much more difficult to regenerate than in ion-exchange chromatography (IEX), hydrophobic interaction chromatography (HIC), reversed-phase chromatography (RPC), etc. Procedures recommended for regenerating fouled sorbents are listed in Table 2.

The validation of the regeneration cycle is not easily accomplished. Often regeneration conditions (choice of cleaning agent, concentration, contact time) are determined empirically. Excessive conditions are frequently applied for obvious reasons of safety in spite of economic effects. The removal of regeneration chemical is also an important issue. That can be easily monitored by sampling a certain volume of the column effluent after re-equilibration and the regeneration compound is analysed by HPLC, GC, amperometric titration, total carbon analysis, amino acid analysis, etc. All rules of cleaning validation can be applied here [107]. An alternative method is a spiking experiment in a scaled-down laboratory column using radiolabelled compounds, if this compound is critical, e.g., because of its high toxicity. As irreversibly bound impurities also influence the separation efficiency and the dynamic capacity, regeneration is indirectly validated by the protein behaviour and the sorption capacity.

Table 2
Procedures recommended for the regeneration of sorbents used for protein chromatography

Procedure	Example of compound commonly used	Remarks
Concentrated salts	NaCl, KCl, $MgCl_2$	Removes all impurities bound by electrostatic interaction, hydrophobic binding is enhanced
Organic solvents	Ethanol, 2-propanol, methanol or acetonitrile together with trifluoroacetic acid[a], ethylene glycol	Removes lipids and other lipophilic substances; often the solubilization effect of ethanol is too low Ethylene glycol is widely used in HIC
Alkaline conditions	NaOH, Na_2CO_3	Hydrolyses a number of natural polymers, proteins, polysaccharides, endotoxins, etc. Na_2CO_3 is a less wetting agent than NaOH
Acidic conditions	Dilute HCl, glycine · HCl buffers, citric acid	Commonly used in immunoaffinity chromatography
Detergents	Sodium dodecyl sulphate (SDS), sodium N-lauryl sarcosinate (SLS), Triton X-100	SLS is a less wetting agent than SDS and the agent is efficiently removed by washing the column with 20–60% 2-propanol in acidic solution
Chelating agents	EDTA, DETAPAC	Very useful for stripping metal chelate columns
Chaotropic agents	Urea, guanidine hydrochloride, KSCN	Solubilizes protein precipitates very efficiently

[a] Not applicable on a large scale although the regenerative power is excellent.

The most widely used method for regeneration of sorbents is with concentrated sodium chloride and sodium hydroxide, as it also affects, to some extent, microorganisms and pyrogens. As far as the purification of an injectable therapeutic agent is concerned, an important rule has to be followed: a sorbent must be used only for one purification step and one product. Validation of regeneration is a very critical aspect, necessary to guarantee a consistent efficiency of treatment in order to avoid separation drifts and product contamination. Using a column for two different products, cross-contamination cannot be excluded. This may be possible in the future, once we have gained more experience in regeneration validation. The stringent rule of one sorbent for one product could then be given up.

4.6. *In situ sterilization*

To avoid microbial cross-contamination, purification has to be carried out under low germ count conditions. A low germ count also prevents the de novo formation of pyrogens. GMP rules require that often the product solution and buffers have to be carefully filter sterilized. The whole chromatographic system cannot be considered as a closed system, because of the presence of valves and ports placed in-line. In situ sanitization or sterilization and storage of the packed column in a preservative solution often combined with regeneration provide low germ counts.

Defining a chromatographic set-up as a closed system is not recommended; it has to be demonstrated, that the closed system is really closed, which looks simple at a first glance but is difficult to prove. As a classical chromatographic set-up cannot be considered as a closed system, chemical disinfection steps have to be defined to keep the germ count as low as possible.

Chemical disinfectants such as formalin, peracetic acid and sodium hypochlorite are commonly used in sterilization industry. The compounds should be used as sterilizing agents as they are adopted frequently in membrane sterili-

zation. Unfortunately, most sorbents are not resistant to oxidizing or alkylating conditions. Therefore, complete sterilisation of columns is not easy. Alternatively, sodium hydroxide solutions are recommended by resin suppliers even though it is not very effective in some instances except at very high concentrations. Most IEX and HIC resins however, possess limited stability in concentrated NaOH and as a compromise up to 1.0 *M* NaOH is frequently used for sanitization and also regeneration. As recently described, the recommended model organism to validate chemical sterilization is *B. subtilis* owing to its resistance to chemicals. The sensitivity of *B. subtilis* in various chemicals has been extensively checked by applying kinetic studies. The inactivation kinetics of *B. subtilis* in 0.05 *M* NaOH are shown in Fig. 5.

Fig. 5 clearly indicates that a long time period is necessary to obtain sterile conditions. For comparison of the efficiency of different inactivation agents, the D_{10} value or K value can be used. Microbial inactivation usually follows first-order kinetics. Assuming that the initial number of microorganisms is N_0 and the number of killed germs after incubation with the sanitizing agent is N_1, following equation can be set up:

$$-\frac{dN}{dt} = k(N_0 - N_1) \tag{14}$$

After integration from N_0 to N and the corresponding times t_0 and t:

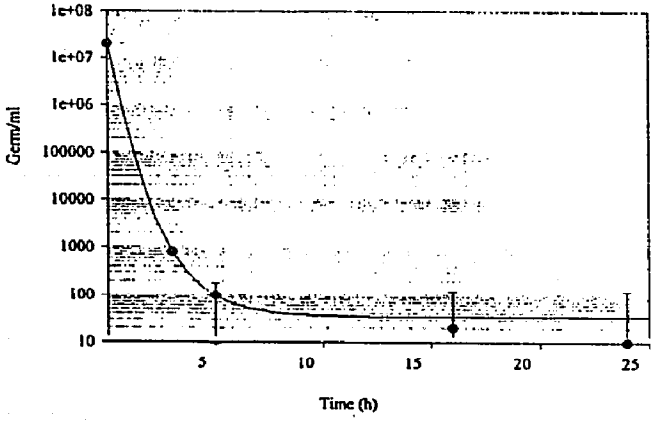

Fig. 5. Inactivation of *B. subtilis* spores by 0.05 *M* NaOH.

$$kt = \ln\frac{N_0}{N} \tag{15}$$

where k is a constant and can be interpreted as the killing rate. A widely used term to evaluate sterilization conditions and time is the D_{10} value, or decimal reduction time. This is defined as the time necessary to reduce the initial germ concentration by one order of magnitude or, in other words, a reduction of 90%. The relationship between k and D_{10} is

$$\ln\frac{1}{10} = -kD_{10} \tag{16}$$

$$D_{10} = \frac{2.303}{k} \tag{17}$$

Although microbial inactivation kinetics do not strictly follow this model, k and D_{10} are widely used. Most common deviations from this model are "time-lag" phases (shouldering) at the beginning of the inactivation and tailing off of a survival curve at longer times. Examples of D_{10} values [27] are shown in Fig. 6.

In the validation of sanitization, the following rules should be followed:

- *Bacillus subtilis* spores should be chosen as a model microorganism, because they are very resistant and easy to cultivate.
- After an inactivation experiment, the inactivation compound must be totally removed or destroyed prior to microbial investigation.
- Germs must be regenerated (grown) at a sub-optimum temperature in order to detect all living microorganisms. Organisms with partial defects can be recovered under sub-optimum growth conditions.

In conventional sanitization protocols, the column is challenged with a certain amount of model organisms. After each step, washing regeneration and sanitization, a sample is collected at the column outlet and a germ balance is calculated [36].

When sterile conditions are necessary and if sorbents are resistant to oxidizing agents such as Q-Hyper D or S-Hyper D, peracetic acid in buffered conditions can be effectively used with no danger of toxicity. It is a very effective

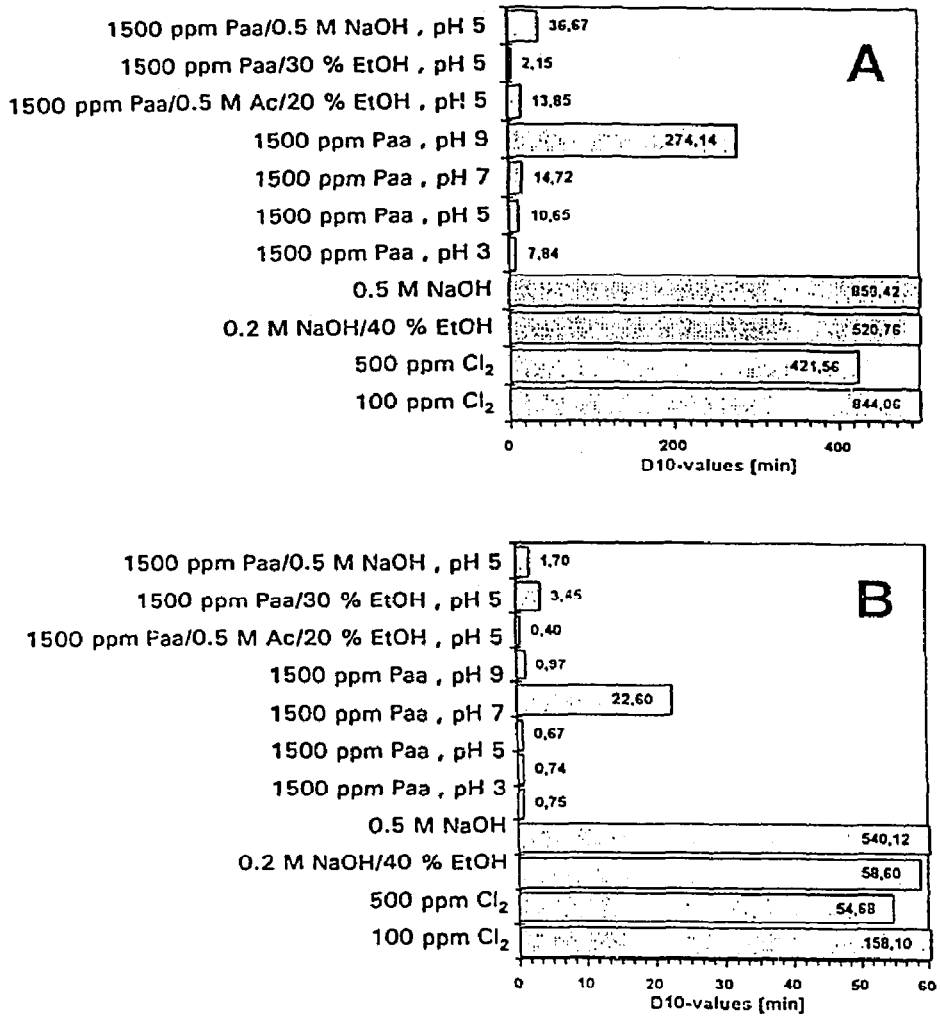

Fig. 6. Comparison of D_{10} values of different agents at (A) 4°C and (B) 25°C affecting spores of *B. subtilis*. D_{10} values were calculated as the average D_{10} values of all D_{10} values calculated between the measuring points of each inactivation kinetics. Survival curves such as shouldering or tailing off were not taken in consideration. Abbreviations: Paa 1500 = 1500 ppm peracetic acid; Cl2 100/500 ppm = hypochlorite, 100/500 ppm available chlorine; 0.5 *M* Ac = 0.5 *M* sodium acetate buffer; 20/30% EtOH = 20/30% (v/v) ethanol.

biocide and it decomposes into non-toxic products such as acetic acid, H_2O and O_2.

A recommended validation protocol for in situ sterilization of an ion exchanger [37] that is used for a down-scaled production column consists of following essential steps:

(1) Wash the column extensively to remove protein and organic material.
(2) Equilibrate the column with buffer containing salts in concentration up to 500 m*M*.
(3) Challenge the packed column with *Bacillus subtilis* spores.
(4) Wash out the bacterial spores.
(5) Displace the washing buffer with the sterilizing solution (e.g., 1500 ppm peracetic acid in acetate buffer), then recycle the santitizing solution, reverse the flow direction at least one time and keep in contact for a few minutes to 30 min.
(6) Ensure that the sterilizing agent is complete-

ly removed from the column by buffer and fill with Caso-bouillon.
(7) Incubate the column at 30°C for 1 week in order to grow possible remaining germs.
(8) Absence of microorganisms confirms a successful sterilization.

Alternatively, one can draw samples from the column outlet, instead of filling the column with Caso-bouillon. This procedure is less sensitive and produces often false negative results.

4.6.1. Problems concerning inactivation of microorganisms

The challenge with *B. subtilis* spores certainly represents the worst case. Sanitisation/sterilization may induce some problems when effected in packed columns:

- The sorbent could not be resistant to the chemical conditions and the optimum concentration and/or time that is necessary for complete inactivation cannot be achieved in a reasonable time.
- Dead ends are present in the column where the sterilizing agent cannot reach the microorganisms in a reasonable time.
- Spores and bacteria tend to form conglomerates and the inner bacteria or spores are protected by the outer layer.
- Some inactivation compounds generate more toxic byproducts.
- The inactivation kinetics often do not follow a strict first-order inactivation rate, grace periods or even activation is observed. Activation can be explained by partial deaggregation of bacteria/spore aggregates.

As a consequence of incomplete sterilization and the fact that a chromatographic column is not a closed system, the column has to be stored in solutions containing bacterostatic agents, such as 20% ethanol, hibitane or sodium azide. It is very important to know that ethanol or hibitane only inhibit the growth of microorganisms and they do not act as inactivation agent [38].

Heat sterilization of sorbents *ex situ*, aseptic assembly of the presterilized column parts and packing of the sorbent under aseptic conditions are also possible strategies to obtain sterile conditions. However, that presumes that separation cycles are all effected under sterile conditions. Column design for *in situ* sterilization in connection with appropriate sterilization protocols seems to be a critical point and a major concern for the future.

4.7. Pyrogen removal

Packed columns are easily contaminated with pyrogens from incorrectly prepared buffers and samples. Pyrogens are generally adsorbed reversibly on the sorbent surface. They can be released during the process and can then contaminate the product. The use of pyrogen-free buffers and equipment is obvious. Certainly products released from *Escherichia coli* and other bacteria contain an excess of endotoxins originating from the host cell itself.

The commonly used method for pyrogen removal in packed columns is washing with NaOH. Pyrogen breakdown under alkaline conditions is shown in Fig. 7.

As pyrogens are negatively charged, anion-exchange chromatography is an excellent method to remove pyrogens from an *E. coli* extract. Up to 2 mg of endotoxin per millilitre can be present in a starting solution after disintegration of *E. coli*. Therefore, removal of endotoxins is a major concern in rDNA protein purification. At least one or two chromatographic steps should be integrated into a purification scheme, which

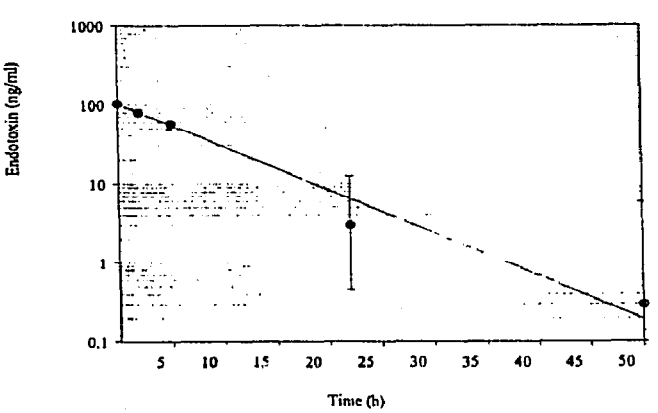

Fig. 7. Endotoxin breakdown by 1.0 *M* NaOH. First-order reaction kinetics can be observed.

allow efficient cleaning with NaOH and/or detergents. Pyrogen removal from sorbents with dilute NaOH and/or detergents used in the final step is mandatory.

Pyrogen breakdown is a slow process. On the other hand, as indicated in Fig. 7, it is less difficult to remove endotoxins from the sorbent instead of destruction in the column. Nevertheless, the last traces of endotoxins remaining after an extensive washing procedure have to be destroyed in the column by means of NaOH or oxidizing agents. The use of endotoxin-free sorbents (some manufacturers offer these), columns, buffers, vessels, etc., are the only way to produce an apyrogenic rDNA product. Relying on the cleaning procedures alone is not sufficient to achieve apyrogenic conditions.

Another agent suitable for endotoxin breakdown is peracetic acid. It acts as a strong sterilizing agent and destroys endotoxin structure and activity.

4.8. Virus removal

The harvest from an animal cell culture can be contaminated by viruses from different sources such as the host cell, the culture medium such as foetal bovine serum and the operator. Validation of virus removal is efficiently done by spiking experiments using appropriate model viruses. The test has to be carried out in scaled-down chromatographic columns, taking into consideration two main questions: how efficient is a given sorbent for the clearance of a particular virus, and how efficient is the cleaning procedure to remove residual viruses from the sorbent?

In the past, a major concern was the model virus for clearance studies. Berthold et al. [39] summarized the selection criteria for model viruses, which are the size of virus, the envelope, the genome structure (DNA/RNA), the strandedness of the genome and the resistance to inactivation. Additionally, other unrelated practical questions are of relevance for production, such as the possibility of achieving high titers, sensitivity and ease of detection. A note for guidance on "Validation of Virus Removal" published by the Ad Hoc Working Party on Biotechnology/Pharmacy [40] describes the relevant criteria for setting up virus clearance experiments.

Viruses used for validation studies are summarized in Table 3 (data from Berthold et al. [39] and Vicari [41]).

A scheme of validation was published by Löwer [42]. He assumed that the inactivation kinetics are independent of the virus titer and that a first-order reaction type takes place (Fig. 8).

The overall reduction factor ($\Sigma_{i=1}^{i=k} R_i$) for virus

Table 3
Examples of viruses that have been used in validation of purification and/or inactivation procedures

Virus	Family	Natural host	Genome	Enveloped	Size (nm)	Shape	Resistances	Experimental titer
Poliovirus, Sabin type I	Picorna	Man	RNA	No	25–30	Icosahedral	Medium	~8.5
Reovirus 3	Reo	Various	RNA	No	60–80	Spherical	High	~8.7
SV 40	Papova	Simian	DNA	No	45	Icosahedral	High	~8.5
Murine leukaemia virus (MuLV)	Retro	Mouse	RNA	Yes	80–110	Spherical	Low	~5
HIV	Retro	Man	RNA	Yes	80–100	Spherical	Low	N.d.
Vesicular stomatitis virus	Rhabdo	Bovine	RNA	Yes	180	Bullet-shaped	Low	~9.5
Parainfluenza virus	Paramyxo	Various	RNA	Yes	150–300	Pleo-spherical	Low	~9
Pseudo-rabies	Herpes	Swine	DNA	No	120–200	Spherical	Medium	~6
IBR	Herpes	Bovine	DNA	Yes	100	Spherical	Medium	~9
Adenovirus	Adeno	Murine	DNA	No	70	Icoshedral	Medium	~6

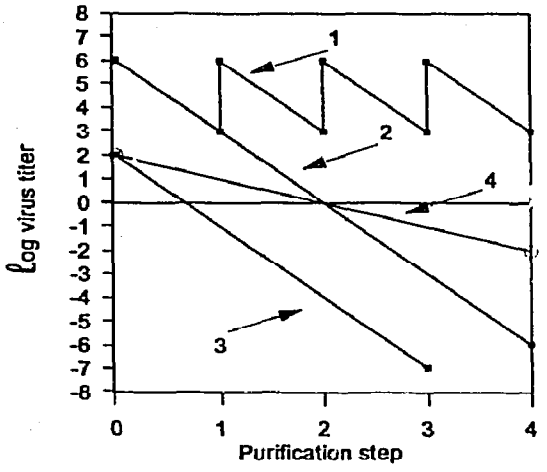

Fig. 8. Validation of virus inactivation. 1 = Reduction of virus titer measured by spiking experiments for each individual step; 2 = cumulative log clearance; 3 = clearance achieved at lower initial titer; 4 = example of insufficient clearance.

removal expressed as the sum of the individual reduction factors (R_i) has to be as high as possible. The following equation represents the calculation of the single R_i values.

$$10^{R_i} = \frac{v' \cdot 10^{a'}}{v'' \cdot 10^{a''}} \quad (18)$$

where v' is the volume before (the initial volume) and v'' is the volume after processing (i.e., harvested elution volume) and $10^{a'}$ and $10^{a''}$ are the virus titers. Generally, two main rules are adopted: first, the respective volume always has to be taken into consideration, and second, the virus titer in the process always has to be measured at the stage where the highest titer is expected. An example of validating the preparation of a clinical monoclonal antibody (Table 4) was given by Mariani and Tarditi [43].

To enhance the virus clearance of a purification process, a virus inactivation step is carried out in addition. Useful methods are solvent/detergent treatment using tri-*n*-butyl phosphate (TBP) together with Triton X-100, deoxycholate or Tween [44], incubation at low pH and heating [45], where applicable.

The validation of cleaning concerning virus removal is a different problem. The sorbent used for virus removal has to be carefully regenerated in order to avoid accumulation of virus in the column, which may be released incidentally.

Table 4
Validation of virus removal by spike-off experiments (data from Mariani and Tarditi [105])

Virus to be removed	Protein A clearance	Hydroxyapatite clearance	Total clearance[a]
Polio Sabin 1	$10^{2.91}$	$10^{6.08}$	$10^{8.99}$ pfu
SV40	$10^{1.63}$	$10^{4.17}$	$10^{5.80}$ pfu
Aujeszky herpes virus	$10^{1.98}$	$10^{4.52}$	$10^{6.50}$ pfu
Moloney murine leukaemia virus	$10^{1.76}$	$10^{8.84}$	$10^{8.84}$ ffu

[a] pfu = Plaque-forming units; ffu = focus-forming units.

The sensitivity of viruses towards regeneration agents such as NaOH and detergents ensures the feasibility of the regeneration process. Sorbents resistant to oxidizing agents (such as Q-HyperD and S-HyperD) can also be treated very efficiently for virus inactivation (diluted solutions of sodium hypochlorite or peracetic acid can be used). An important unanswered question remains, however, because residual virus cannot be easily detected in a column like bacteria, but the same problem arises as described with bacteria. Dead ends are present in conventional columns, which may act as a trap for viruses.

As a conclusion, it can be stated that chromatographic methods are an effective way to remove viruses from an rDNA product. This approach, however, necessitates virus removal from the sorbents, which can be achieved with various chemical agents that inactivate the virus whatever the origin of the contamination. The use of defined viruses or validation models is now cleared, and the reduction factors have to be as high as possible according to the context.

4.9. DNA clearance

A particular aspect of column regeneration is DNA removal. It is known that biotechnology-derived products may be contaminated with DNA and DNA fragments generated during the cell cultures. The limits of admitted contamina-

tion are very stringent as required by regulation for the production of medicinal products derived from biotechnology. DNA removal is an aspect of the problem recently discussed in a specific review [46]. Different methods for DNA reduction by chromatography are known, such as ion-exchange chromatography, size-exclusion chromatography, affinity chromatography and hydroxyapatite chromatography. Size-exclusion chromatography is based on the general assumption that DNA molecules are larger than proteins. However, this does not apply to small fragments of DNA. Cation-exchange resins such as DEAE or Q ion exchangers are good means of decreasing DNA levels in biological material, because of its strong affinity for charged surfaces. The strength of DNA adsorption depends on the molecular size of DNA [47]. Elimination of DNA tightly adsorbed on cation-exchange resins could create a problem. Highly concentrated salt solutions (e.g., 2 *M*) have been suggested but are not a guarantee that all DNA molecules are eliminated. In this situation, chemical hydrolysis of remaining DNA on the resin could be appropriate. It has been reported [27] that peracetates at 100–500 μg/ml concentration are very effective for destruction of DNA molecules, owing to the very high oxidizing power. This property could be advantageously used to clean columns but assumes that resins are stable in an oxidizing environment. When the feedstocks are assumed to contain large amounts of DNA, immunoaffinity chromatography can be used to adsorb the target protein whilst DNA is removed in the flow-through of the column or in the wash. This operating mode demonstrated that more than 99.99% of radiolabelled DNA was removed [46].

Speculating on the configuration of a chromatographic process for the separation of a protein with a high level of DNA removal, a suggestion could be first to use immunoaffinity chromatography for efficient capture of the target protein followed by an ion-exchange step using a Q resin. The first column would eliminate a large amount of DNA in the wash while the remaining traces of DNA could be adsorbed on the cation-exchange resin. This would then be treated later with a buffer solution containing up to 5000 ppm of peracetic acid.

An example of a spiking experiment is shown in Fig. 9. Host cell DNA was prepared by feeding the cell line with radiolabelled nucleotides. The DNA was extracted from the cells and a cell culture supernatant was spiked with it.

If spiking experiments are carried out to

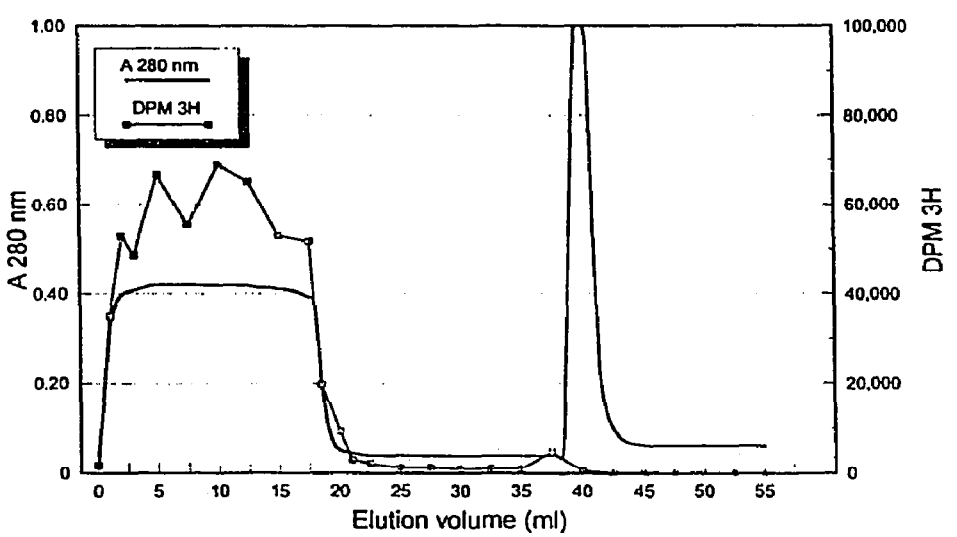

Fig. 9. DNA clearance validation experiment using ^{32}P-labelled DNA produced by feeding the cell line with radiolabelled nucleotides. The prepurified cell culture supernatant from a hybridoma cell culture was spiked with DNA and applied to a protein A column. A clearance of about 3 log steps can be achieved.

evaluate the clearance factors, one has to ensure that the DNA has the same physico-chemical nature as the DNA present in the feedstock.

5. Degradation of chromatographic sorbents

Packing materials for column liquid chromatography designed for protein separation have a limited lifetime. This is due to two main reasons: (i) physico-chemical deterioration such as changes in temperature and pressure, changes in pH and ionic strength and washing in the presence of dissociation agents; (ii) biological deterioration related to enzymatic hydrolysis and fouling are also responsible for the progressive decrease of their effectiveness. Degradation can result in the release of chemical compounds from the packing material when hydrolysis occurs. Chemical aggressions are generally at the basis of hydrolysis of the polymeric material and of the chemical link between a ligand and the matrix.

Cleaning and sanitization operations described earlier prevent foulant building up with a clear benefit for the user. However, they can have a deleterious effect on the packing material itself. Even limited chemical modifications could diminish ligand selectivity, introducing frequently unwanted non-specific adsorption effects.

Degradation of chromatographic sorbents is not only a problem as regards the lifetime, but also because of the release of chemical compounds that can contaminate the purified biological compounds to some extent. This situation has induced suppliers and users to define procedures of cleaning and sanitizing to prevent the generation of undesired by-products. Criteria to define cleaning procedures and analytical techniques have to be characterized by a high level of specificity and selectivity, particularly when purified biomolecules are intended for human use.

Although no precise rules exist, acceptable assay detection limits are in the range 1–100 ng of released material per mg of biologicals [48]. This level of acceptance depends, however, on a number of factors, such as the toxicological danger of the released compound and the purification stage of the biomolecule itself. If this is an early stage of the purification, higher contamination could be tolerated provided that the subsequent steps contribute to removing the contaminants. It must be noted that contamination of biologicals can also occur when chemicals and by-products from the sorbent synthesis are not totally eliminated and when chemical cleaning agents subsequent to a regeneration–sanitization operation are not completely removed.

Although it is admitted that all sorbents can, under particular circumstances, release chemical compounds subsequent to the matrix hydrolysis, it is well known that affinity media are of the most concern. They are generally the result of a special chemical treatment (activation) followed by immobilization of the ligand; the stability–instability of the linkage between the ligand and the matrix is the best known source of leakage [49–51].

Mechanisms resulting in the generation of chemical molecules released into the mobile phase in a chromatographic column are classified according to the zone where they occur. The generally admitted mechanisms of leakage are partial matrix hydrolysis, release of entrapped material into the polymeric network, hydrolysis of the attachment point between a ligand and the matrix, dissociation of a non-covalently bound ligand and subunit dissociation of the ligand molecule or its partial hydrolysis. Degradation of covalent bonds can occur during redox reactions or nucleophilic attacks. Slow release of material may induce, owing to special local conditions, the formation of molecular aggregates that can be easily entrapped. Conditions of solvation of these aggregates with aqueous or aqueous–organic systems may then provoke progressive disaggregation with consequent leakage.

5.1. Extractables

Extractables are defined here as soluble chemicals that are not totally eliminated from the sorbent manufacturing process. They can be constituted of linear polymers (e.g., polysaccharides), monomers (e.g., acrylamide deriva-

tives that are not completely converted into polymers), cross-linkers, catalysts, solvents and by-products. Additionally, affinity sorbents may contain activating agents in excess (or their degradation products), unreacted ligands and blocking agents.

Commercially available process media are based on different chemistries [52] and, as such, they may contain compounds of different nature. Two main examples are illustrative of the situation: (i) polysaccharide-based sorbents that are stabilized by cross-linkers and (ii) synthetic material resulting from a three-dimensional polymerization of monomers. In the first case traces of cross-linker should be assayed after having submitted the chromatographic sorbent to intensive extraction in aqueous and organic media. Hot water under agitation is a possible approach to dissolve water-soluble molecules; water–alcohol mixtures and pure ethanol are also used to dissolve molecules that are not very soluble in water. In some instances non-polar solvents are used in the assay of traces of material utilized during bead formation in emulsion polymerization [53].

Identification of tracked material is not an easy step and can be simplified only when a detailed description of the chemical synthesis of the sorbent is known. As a first step, analytical HPLC can be an affordable and sensitive means to identify and quantify extracted chemicals (see below).

Fig. 10 shows HPLC profiles of extracted DEAE-Spherodex to quantify the residual amount of butanediol diglycidyl ether used as in situ cross-linker to stabilize the organic network. Establishment of the retention time of this molecule and quantification were performed by spiking the extracted and concentrated solution with known amounts of standard solutions of butanediol diglycidyl ether. By using internal standards, if applicable, accurate calibration graphs can also very easy be established.

In the second case, acrylic monomers are currently measured in finished packing material. This can be approached similarly by submitting the solid phase to intensive extraction with the most appropriate solvents for the investigated

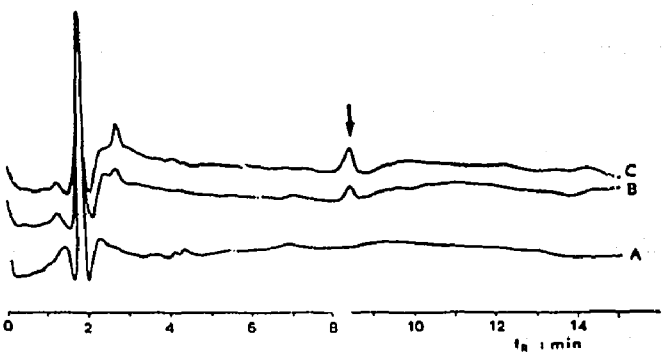

Fig. 10. Determination of traces of cross-linking agent (butanediol diglycidyl ether, BDDGE) possibly present in the supernatant of DEAE-Spherodex stored in 1 *M* sodium chloride. (A) Chromatogram of whole supernatant; (B) supernatant (A) spiked with 12 ppm of BDDGE, (C) same as (A) spiked with 24 ppm of BDDGE. The arrow indicates the retention time (t_R) of BDDGE. HPLC was performed using C_8 reversed-phase silica of 5-μm particle size; elution (isocratic) was carried out with water–acetonitrile (60:40, v/v); flow-rate, 1.0 ml/min; UV detection at 210 nm.

monomer followed by HPLC analysis. Sodium acrylamidomethylpropanesulphonate, extensively used for the preparation of strong cation exchangers [54], is illustrative of the situation. This monomer is very soluble in water with which it is extracted for several hours at 25–70°C; the solution is concentrated, spiked with different amounts of standard monomer and analysed by HPLC. Under the conditions described in Fig. 11 the sensitivity of the assay is 0.25 ppm.

The preparation of beaded sorbents is carried out by suspension–emulsion polymerization and/or cross-linking in the presence of non-polar solvents used as a "vehicle". The recovered beads can therefore contain traces of this organic solvent and emulsifiers that can be easily and rapidly quantified. When present, they generally are not water soluble. However, traces can be found when packed columns are treated with more or less polar organic solvents. One easy way to quantify these compounds is illustrated in Fig. 12. Pharmaceutical paraffin oil used for the preparation of Trisacryl beads is quantified by extraction with a chlorinated solvent such as carbon tetrachloride and paraffin oil is identified by infrared analysis. Quantification is effected by

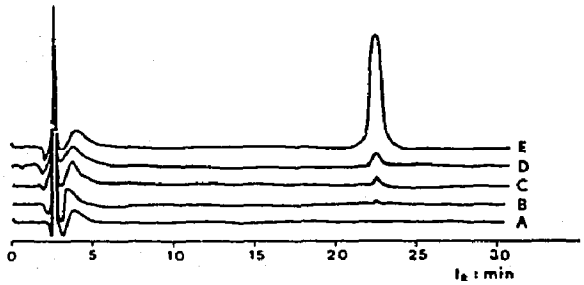

Fig. 11. Determination of traces of unpolymerized sodium acrylamidomethylpropylsulphonate (AMPS) possibly present in the supernatant of S-HyperD stored in 1 *M* sodium chloride. (A) Whole supernatants; (B), (C) and (D) supernatant (A) spiked with 1, 2 and 4 ppm, respectively, of fresh AMPS; (E) injection of 12 ppm of pure AMPS. HPLC was performed using C_{18} reversed-phase silica of 5-μm particle size; elution (isocratic) was carried out with water–trifluoroacetic acid (99.9:0.1, v/v); flow-rate, 1.0 ml/min; UV detection at 215 nm.

comparison with standard solutions of pure paraffin oil.

Most chromatographic media are supplied as aqueous suspensions containing 20% ethanol or 0.02% sodium azide to prevent bacterial growth. Elimination of this bacteriostatic material, classified among "extractables", is not an obvious operation. Total removal of ethanol, for instance, requires extensive column washing, which depends on the pore size of the sorbent [55]. Large particles with small pores are more difficult to clean than small particles with large pores [56]. It is not uncommon to wash with more than ten column volumes to lower ethanol and sodium azide contents to below ppm levels in the eluate.

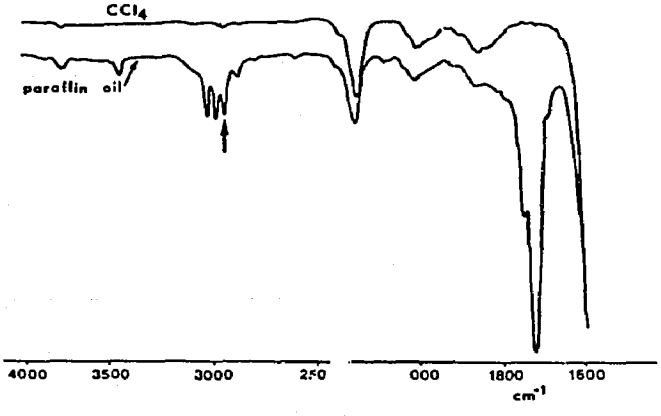

Fig. 12. Typical IR spectrum of paraffin oil for the quantification of traces of oil present in synthetic beads. Beads were first extracted extensively with carbon tetrachloride and the solution was filtered, concentrated and submitted to IR analysis. Absorbance of the peak indicated by the arrow compared with a calibration graph was used for calculation.

5.2. Degradation products

Chemical deterioration of packing materials affects their long-term performance for protein separation. Many agents can be responsible for the degradation of the polymeric network used before column packing or as part of the fractionation process (regular elution chemicals or cleaning–sanitizing solutions).

To illustrate clearly this situation, two main aspects of sorbent degradation have to be considered: (i) chemical degradation of the polymeric matrix and (ii) degradation of affinity-related chromatographic media subsequent to ligand release.

Adsorbent particles may be sensitive to a number of external agents that contribute to modifying the behaviour of the sorbent and to creating degradation products. Chemical, physical and biological attack are of special concern for the integrity of the polymeric matrix.

5.2.1. Chemical damage

Although chromatographic materials are generally chosen to be totally insoluble and unreactive towards common chromatographic eluents, they may be sensitive to other chemicals employed only in some circumstances. The sensitivity towards chemical agents depends on the nature of the solid matrix. A classification of cleaning agents according to the nature of the sorbent is given in Table 5.

Hydrolysis of matrices generally occurs at extreme pH values according to their chemical nature. Polyacrylamide and some polyacrylamide derivatives are partially hydrolysed in strongly alkaline solutions (e.g., 1 *M* sodium hydroxide), with a release of side-chains, whereas the backbone is stable. Protected polyacrylamide derivatives are, however, stable under such conditions for an extended period of time [57]. In acidic

Table 5
Recommended cleaning–sanitizing agents according to the chemical nature of packing material

Sorbent nature	Recommended cleaning agents	Comments	Ref.
Polysaccharide	Sodium hydroxide		106–110
	Mercury salts (merthiolate)		
	Glutaraldehyde	Formation of acetals	
	Hibitane digluconate in ethanol	Ethanol concentration below 25%	
	8 *M* urea	with cross-linked agarose	
	4 *M* urea with dilute sodium hydroxide		
	Propylene oxide	May react in alkaline conditions	
Polyacrylamide derivatives and Polyacrylates	Ethanol–acetic acid	Ethanol concentration below 30%	36, 111
	Hydrochloric acid		
	Sodium hydroxide	Only with stabilized derivatives	
	Peracetic acid	To be avoided with OH-containing polymers	
Polystyrene derivatives	Ethanol–acetic acid	May swell packings	
	Hydrochloric acid		
	Sodium hydroxide		
	Glutaraldehyde		
	Urea in any concentration		
	Ethanol	May swell packings	
	Peracetic acid	To be avoided with cation exchangers	
Silica	Ethanol	Any concentration	107–111
	Ethanol–acetic acid		
	Strong acids		
	Mercury salts		
	Glutaraldehyde		
	Urea	Any concentration	
	Hibitane in ethanol		
	Peracetic acid		

conditions all polyacrylamide matrices are generally stable.

Conversely, polysaccharides are more sensitive to acidic conditions, with hydrolysis of glycosidic bonds that form the backbone of the matrix. Agarose-based sorbents, for instance (both in its native and in cross-linked form) can be completely dissolved in warm acids in few hours; they release carbohydrate fragments when at pH <3. Under alkaline conditions, polysaccharide-based sorbents are more stable; however, complex hydrolytic processes occur with the formation of a wide range of compounds (Fig. 13). Calcium ions, in addition to oxygen, influence polysaccharide degradation, the general outcome of which is depolymerization of the chains. Alkaline washings recommended for cleaning such sorbents between runs may cause, however, only limited hydrolysis, with the progressive formation of small amounts of polyhydroxy acids. Polyesters and polyamide-based resins are also sensitive to strong acids and strong alkalis [58].

Oxidizing agents are particular degrading compounds for a number of sorbents, especially in the presence of transition metal ions [59]. Fragments of polymeric chains can be generated from ionic polystyrene resins under mild oxidizing conditions [60]: phenolsulphonic acid, sulphobenzoic acid and esters of soluble sulphonated oligomers can be released. Sorbents with hydroxy groups, such as polysaccharides, are

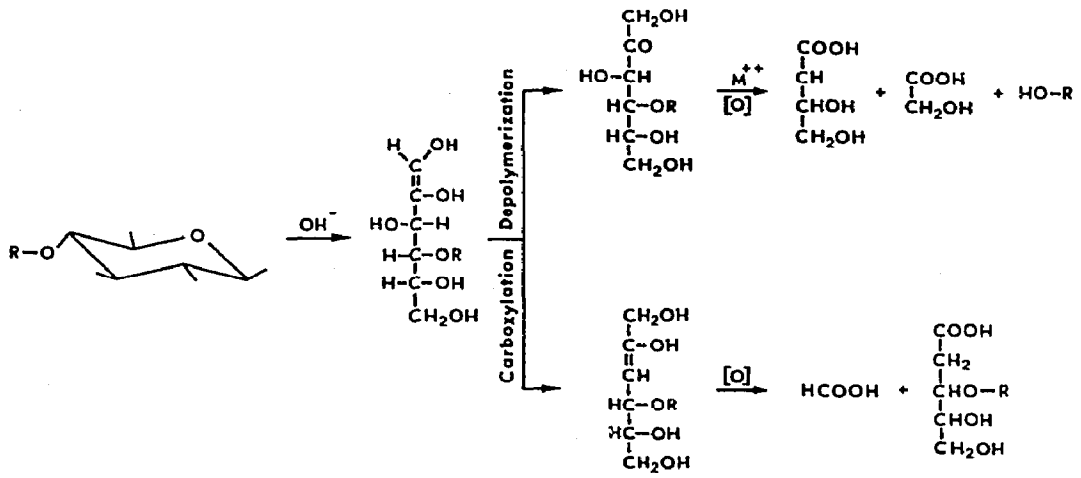

Fig. 13. Hypothesis of chemical degradation of a polysaccharide R by its terminal sugar under alkaline conditions (OH^-) and catalysed by oxygen (O) and transition metals (M).

particularly susceptible to oxidation; osidic linkages can be destroyed and sugar rings opened with the formation of carbonyl groups. This reaction is well known for ligand coupling in affinity chromatography [61]. A high carbonyl content subsequent to a substantial degradation can induce a loss in bead structure. Over-oxidation structures have been described for cellulose when treated with periodates [62].

As described in a previous section, in order to sterilize spore-contaminated sorbents, oxidizing agents should be used. A stability study showed that with special polymers peracetates can be used extensively for a large number of runs with no loss of sorbent properties [37]. With polysaccharide-based sorbents and other hydrophilic sorbents, the use of oxidizing agents seems to change irreversibly the ion-exchange capacity of the resins and the chromatographic properties [63].

5.2.2. *Physical damage*

Most chromatographic packing materials are stable towards heat. They can be heated at 120–130°C in an autoclave or cooled to −20°C with no damage to their chemical structure and chromatographic behaviour. Non-cross-linked agarose beads can be damaged at temperatures above 40°C by disrupting hydrogen bonding between chains. Higher temperatures can damage quaternary ammonium groups when exposed in their hydroxide form.

Generally, radiation does not damage a synthetic matrix or only to a very limited extent, except when amino groups are present on the resin. Radiolytic action is more effective with polysaccharide-based supports, especially when they are in dry form [64].

Compression, attrition and shrinking–swelling can damage chromatographic packings without real denaturation of their chemical structure. These physical treatments generate small fragments with an impact on column flow-rates and back-pressures. This phenomenon is particularly known for hydroxyapatite crystals and is a major factor impeding the large-scale utilization of this sorbent. A way to circumvent physical damage of hydroxyapatite crystals is embedding polysaccharide beads or polymer beads.

With soft chromatographic packings, compression may cause cracks that modify the column performance. In contrast to chemical damage, physical forces do not generate chemical compounds that may partially contaminate the purified biologicals. However, with macroporous resins obtained by aggregation of submicroscopic particles it is possible to generate very small solid "pieces" of polymers in metastable suspension that can be classified among contaminants difficult to separate even by centrifugation.

The stability of packing materials when submitted to mild ultrasonic treatments is also very different from sorbent to sorbent. Generally, sorbents are very stable when they are constituted of soft or semi-rigid polymers. Silica-based sorbents are also fairly stable in spite of their rigid structure; macroporous polystyrene-based material is, however, unstable, creating a milky white suspension of submicroscopic particles resulting from the disaggregation of the original macroporous structure [63].

5.2.3. Biological damage

As far as the biological degradation by means of enzymes is concerned, only polysaccharide material can be affected whereas synthetic and mineral supports are obviously stable. Dextranases, agarases and cellulases degrade, respectively, dextran-based sorbents, agarose beads and cellulose fibres or beads in native and, in limited extent, cross-linked forms.

5.2.4. Special focus on affinity media

Degradation of affinity chromatographic media with subsequent release of soluble structures is one of the major aspects of liquid chromatography to be studied. As a general rule, a covalent chemical bond has to be formed to attach the ligand to the polymeric structure of the matrix. Nevertheless, even when the ligand is properly attached by chemical bonds, leakage is observed and is mainly dependent on the nature of the activation reaction and the physico-chemical treatment of the immobilized ligand. Three major mechanisms of ligand release directly related to the ligand itself have been described: cleavage of the covalent bond, dissociation of subunits constituting the ligand and partial hydrolysis of the ligand. Other general mechanisms of ligand leakage exist but they are associated with the matrix as described above. In certain cases the released molecules are structurally different from the ligand; these structures must be known to define an appropriate assay for the quantification of the leakage phenomenon and also to perform toxicological studies when needed. Nucleophilic attack is one of the most important causes of ligand leakage. Potentially it increases when the asymmetry of the carbon bond to an adjacent atom increases and becomes more dipolar. The presence of an atom with withdrawing properties such as oxygen, sulphur or nitrogen next to a carbon atom increases the risk of a displacement reaction with consequent ligand leakage. Accessibility to the chemical bond is, however, of importance for the hydrolysis of the linkage. For instance, large macromolecules immobilized with chemical groups close to their surface provide steric protection and thus improve bond stability.

Subunit dissociation of particular macroligands has been evidenced. For instance, dissociation of concanavalin A subunits in the presence of chaotropic agents [65] can result in a loss of partial structure that may contaminate the purified protein. All multimeric ligands involving subparts in their structure that may be dissociated reversibly or irreversibly (e.g., LDH, antibodies) represent a potential danger of leakage that must be monitored. Proteinaceous ligands are also very sensitive to proteolytic attack by proteases present in the feedstocks. This is the case with immobilized lectins, protein A, protein G and immobilized antibodies. Peptidic fragments of various nature can be generated with two consequences: (i) the risk of contamination of the purified biological and (ii) degradation of the effectiveness of the chromatographic packing. Monitoring and quantification of released material in these circumstances are serious problems that must be addressed case by case.

A particular example of the degradation of an affinity-related packing is given by immunosorbents [66]. The decrease in binding capacity for a given antigen during use seems related to the nature of the eluting agent or cleaning conditions. Acidic solutions are responsible for a progressive decrease of the adsorption capabilities of the antibody for the antigen. Potassium thiocyanate in some instances is responsible for the rapid degradation of the immobilized antibody [67]. Partially denatured and unfolded protein domains are more easily accessible for proteases than native compactly folded proteins. Additionally, the degradation rate of an im-

munosorbent seems to be more rapid when the amount of coupled antibody is high. Antibody leakage and non-specific adsorption of contaminants (except proteases) do not seem to be responsible for significant losses of antibody activity during intensive and prolonged use.

5.3. Quantification of released material from chromatographic sorbents

The determination of a small amount of released material in column effluents is frequently an important issue. Identification leached material obviously simplifies the analysis and is a compulsory step in the strategy of quantification of released material. Leached material should be measurable in the presence of purified biologicals with which they could have common features. This is the case when determining immunosorbent leakage or protein A leakage, both protein-based ligands, among purified target proteins. As stated before, quantification of released material regardless of the origin (residual chemicals from sorbent synthesis or hydrolysis products from the polymeric support or from the ligand) is necessarily preceded by its chemical identification.

A number of analytical approaches exist that are applicable both in static experiments and in on-column experiments. Static experiments, most frequently adapted to the stability of the matrix, consist of incubation of the sorbent in the presence of solutions at extreme pH at room temperature for several hours to hundreds of hours. An increase in temperature may also help into the simulation of the sorbent ageing. Release kinetics can be studied in this way. On-column trials are defined to identify formally the leakage phenomena under real working conditions in the presence of the separated protein and at various stages of the chromatographic cycle. Cleaning solutions could lead to sorbent degradation, which is not necessarily a problem if the released material is flushed out and if the column behaviour remains unchanged. The main disadvantage of proteinaceous ligands intended for purification of injectable therapeutics is the regulation of their preparation; the protein ligand should in fact be produced in the same regulatory frame as the product itself concerning purity, identity, potency and consistency criteria.

5.3.1. Gravimetric monitoring

Extraction of resins with appropriate solutions is likely to give an indication of the extent of leakage after solvent evaporation under different conditions with the possibility of performing kinetic studies. This approach is unspecific and also the least sensitive. A standard test exists for the acceptability of ion-exchange resins intended for the food industry [68] which involves extractions in distilled water, 15% ethanol in water and 5% acetic acid. In all instances the released material must not exceed 1 ppm.

5.3.2. Chromatographic methods

Most generally, HPLC provides a convenient method of separating compounds simultaneously released from a sorbent. It also provides sensitive detection when coupled with UV adsorption, refractive index and fluorescence detection. Assumptions must be made involving the comparative behaviour of the unknown leached material with a reference standard. With simple ligands such as hydrophobic groups (e.g., phenoxypropyl residues as degradation products from Phenyl-Sepharose [69]) or dye ligands [70,71], it is possible to define standard compounds (see Fig. 14). When degradation products are derived from macroligands or complex pathways of sorbent degradation, HPLC provides only quantitative chromatographic spectra and does not indicate the nature of each peak.

Volatile compounds from column degradation could constitute a technical difficulty for analytical determinations. In this case gas chromatography represents a powerful method for detection, quantification and also identification if coupled with mass spectrometry. The sensitivity could reach ng/l levels. Volatile amines such as tetramethylethylenediamine released from DEAE-Sepharose Fast Flow when exposed to extreme pH, have been determined by gas chromatography [55]. Analytical HPLC is very useful for quantifying easily residual material from sorbent synthesis [72]. Cross experiments by

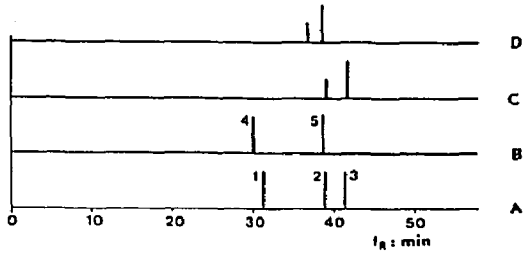

Fig. 14. Typical retention times (t_R) determined by HPLC of (A and B) dyes used as ligands and (C and D) leached dyes obtained from a Trisacryl matrix treated under strongly alkaline conditions (1 *M* sodium hydroxide at 37°C for 7 days). The dyes studied were Reactive Blue-2 and Reactive Red-120 and their derivatives. 1 = Deactivated Reactive Blue-2; 2 = ethylenediamine-condensed carboxylic derivative of Reactive Blue-2; 3 = carboxylic derivative of Reactive Blue-2; 4 = deactivated Reactive Red-120; 5 = carboxylic derivative of Reactive Red-120. HPLC analysis was performed using a C18 reversed-phase silica column (250 mm × 4.6 mm I.D.) using a two-step linear gradient. The first was with acetonitrile from 0 to 15% in 0.5% triethanolamine–1% acetic acid in water for 2 min and the second was with acetonitrile from 15% to 50% for 57 min.

varying stationary phases and solvent systems should be made to identify peak overlaps [73].

5.3.3. Chemical assays

Degradation of polysaccharide-based sorbents with the release of small-sized compounds, and also the leakage of sugar-like ligands, can be detected and quantified by using specific colorimetric hexose assays. Sulphur-containing leachates can be followed by sulphur determination and other organic molecules by nitrogen determination.

A number of colorimetric assays are available for quantifying leached material of protein origin. Lowry's chemical method is based on the detection of phenol side-chains of tyrosine residues, while Bradford's assay is based on the colour of Coomasie Brilliant Blue after interaction with proteins. The sensitivity of these methods can reach the microgram level [74]. Colloidal gold methods, which are based on gold sol aggregation in the presence of proteins with a colour change from red to blue, can also be used to detect traces of proteins [75]. Leached proteins or protein fragments can alternatively be quantified by classical amino acid analysis after total hydrolysis. Release of mineral compounds, such as silicon, from silica-based sorbents, aluminium from alumina-based material [75] and calcium from hydroxyapatite-based sorbents can be easily quantified by elemental analysis and specific colorimetric methods.

5.3.4. Spectroscopic methods

Identification of leachates can be carried out by ^{1}H NMR spectrometry. Investigations in this field, for instance, demonstrated that with the leakage of ligands when the sorbent is incubated under extreme conditions a significant part of the hydrolysed matrix is also present. Strong signals have been shown between 2 and 5 ppm, characteristic of glycosidic groups in a fraction of dyes leached from a dye sorbent stored under acidic conditions [82]. A similar conclusion has been reached with phenylagarose when stored in the presence of 0.1 *M* hydrochloric acid: a signal at 2–5 ppm attributable to a saccharidic material was associated with the signal of phenyl ligand protons at 6.8–7.2 ppm [76].

For convenient interpretation of ^{1}H NMR signals, samples must be concentrated and, when composed of various chemicals, they must be separated. It has been reported [8] that ^{1}H NMR could be used for the identification of 5-(hydroxymethyl)-2-furaldehyde leached from S-Sepharose Fast Flow stored under acidic conditions. This type of sorbent can in fact turn yellow when treated first with sodium hydroxide and then exposed to acidic conditions, corresponding to the formation of furan derivatives when sugar is degraded [76].

5.3.5. Immunochemical methods

The availability of specific antibodies against leached material offers the possibility of detecting very specifically trace amounts of material even in the presence of other chemicals or biologicals. Antibodies should possess a dissociation constant of immuno complexes smaller than that related to the interaction between the molecule of interest and the leached ligand. This condition, which is frequently satisfied, is im-

portant as the protein may interact with the epitope of the ligand responsible for the antibody recognition.

Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are both used for these determinations. The detection of low leakage levels of antidigitoxin using RIA has been reported [77]; ELISA has also been used for years to detect leakage of material from chromatographic sorbents. Antibody leakage from sorbents was detected in 1986 with a detection limit of about 10 ng/ml [62]. ELISA utilizing avidin–biotin as a detection system also provided a powerful assay to detect immunoglobulin G leakage from immunosorbents at a level of about 0.5 μg/ml [78]. Nowadays 0.1 ng/ml detection levels can be reached routinely with immunoassay methods [79]. ELISA-based assays can not only be used for the specific detection of large and native antigens; fragmented antigens with the intact recognition epitope can still be assayed with a similar level of sensitivity [80]. This is extremely important because large ligands, such as proteins (lectins, antibodies, antigens, protein A), can be released in small fragments if proteolytic enzymes are present in the sample to be purified. In this respect ELISA, in addition to RIA, should be performed with polyclonal instead of monoclonal antibodies. The detection, for instance, of traces of protein A (native or fragments) leached from protein A sorbents that can contaminate antibody preparations is of great importance when antibodies are intended for in vivo diagnoses or for human therapeutics. Comparative studies have been conducted to determine the level of protein A leakage from different commercially available sorbents [81]. To monitor protein A column leaching, two powerful ELISA-based assays have been described [82]. The first involves rabbit antibodies against protein A for capturing and anti-protein A F(ab)2 antibodies conjugated with horseradish peroxidase for detection. The second approach involves an anti-protein A–biotin conjugate permitting an increase in the sensitivity of the assay to the subnanogram range. This level of detection is very important with antibodies purified on protein A resins and intended for massive therapeutic use.

ELISA-based assays can also be easily used for detecting very low levels of small ligands released from the matrix. A particularly well documented example is the quantification of traces of dye [83–86] using polyclonal antibodies prepared after conjugation of the dye on KLH and injection into rabbits.

It has been demonstrated that the sensitivity of these assays was 100–10 000 times higher than that of a classical spectrophotometric assay and was modulated by chemical substituents on the dye molecule. No cross-reaction occurred between different anti-dye antibodies, showing the high specificity of the assay in spite of the close chemical structure of the dyes.

Fig. 15 shows ELISA assay curves obtained with two dyes classically used in affinity chromatography. Dye traces could also be detected and quantified in the presence of the protein separated using the immobilized dye itself, demonstrating that the dye–molecule interaction does not constitute an obstacle to immunoenzymatic assay. This can be attributed mainly to the high affinity constant between the antibody and the dye. Fig. 16 gives two examples of the correlation of the dye immuno quantification results with the real amount of dye present in an aqueous solution containing the protein possessing a known affinity for the dye. This correlation has been verified with a number of proteins in the presence of various amounts of dye in solution.

Table 6 summarizes some inhibition reaction results using proteins of different molecular mass, isoelectric point and affinity for the dye. It seemed clear this immunoenzymatic assay could be applied in the presence of different proteins with no loss in its specificity and sensitivity in a range of protein concentrations between 1 and 10 000 μg/ml and of dye concentrations between 1 and 500 ng/ml.

5.3.6. Radiochemical labeling

Macromolecular ligands as well as low-molecular-mass ligands can be radiolabeled and the

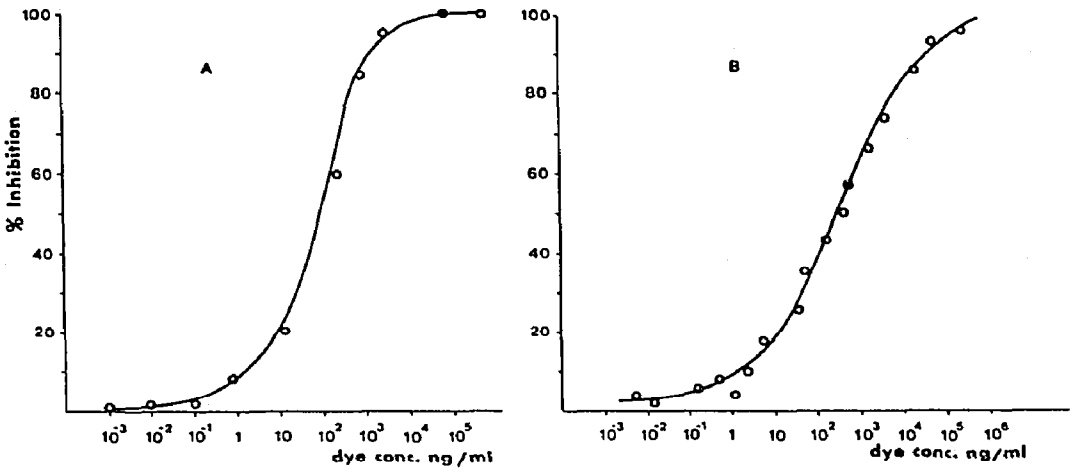

Fig. 15. Immunoenzymatic competitive inhibition assay curves obtained with (A) Reactive Blue-2 and (B) Reactive Red-120 used as ligands for affinity chromatography. Quantification was performed using anti-dye specific antibodies in microtitration plates with bovine haemoglobin–dye conjugates as a challenge for wells coating (for details see specific references).

leakage level measured directly in the column effluents. This method is extremely sensitive but it suffers for some practical limitations. First, it cannot be used to monitor operational ligand leakage since radiolabeling of the ligand is not permitted for the separation of the final product. This method can only be used as a reference for validation studies and then extrapolated to the real affinity chromatography case, assuming that leakage mechanisms remain similar.

Additionally, when facing macromolecular ligands (e.g. proteins such as antibodies), leakage of "pieces" of leached molecules that are not labeled, cannot be detected.

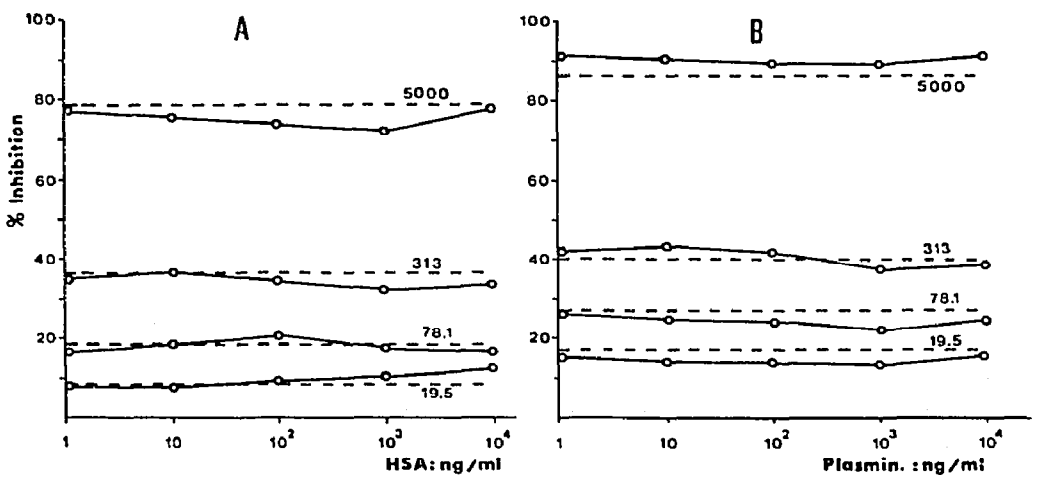

Fig. 16. Examples of correlation of dye ligand quantification in the presence of affinity proteins separated using immobilized dyes themselves. (A) Quantification of different amounts Reactive Blue-2 in the presence of human albumin (HSA) at different concentrations; (B) the same applied to Reactive Red-120 in the presence of plasminogen at different concentrations. Broken lines indicate the real concentration of the dye (19.5, 78.1, 313 and 5000 ng/ml) in the protein solutions. The continuous lines show the real experimental results. Quantification was affected with a competitive inhibition ELISA-based assay using specific anti-dye antibodies.

Table 6
Immunoenzymatic competitive inhibition assay of Reactive Blue-2 in the presence of various proteins

Protein	Molecular mass	Net ionic charge at neutral pH	Origin	Inhibition reaction (%)
No protein	N.a.	N.a.	N.a.	21.0
Albumin	68 000	Anionic	Human	17.5
Albumin	67 000	Anionic	Bovine	15.1
β-Lactoglobulin	36 000	Anionic	Bovine	15.5
Alcohol dehydrogenase	120 000	Cationic	Yeast	12.4
Glutamate dehydrogenase	330 000	Anionic	Bovine	12.9
Lactate dehydrogenase	140 000	Anionic	Porcine	28.5
Malate dehydrogenase	70 000	Cationic	Porcine	15.9

Dye concentration = 50 ng/ml; protein concentration = 10 ng/ml; N.a. = not applicable.

6. Toxicity of degradation products from sorbents

Chromatographic packings used repeatedly for preparative purposes have a limited lifetime, depending essentially on the severity of the cleaning in-place operations. As stated earlier, foreign chemical material that can come from solid phases represents a risk of contamination for the biological to be purified. Toxicity data for related material could constitute a key issue and a discrimination parameter for the choice of chromatographic packings. As the released chemicals are not necessarily those expected (a released ligand is not chemically identical with the ligand prior to immobilization), a formal identification is necessary. Toxicity studies in vivo and/or in vitro then follow.

6.1. Identification of released material

Chemicals originating from the sorbent synthesis that are still present as a result of uncompleted reactions or inadequate washings can be easily identified and quantified. When in the presence of synthetic sorbents, monomers are usually sought. Most generally, toxicity data on monomers exist in the literature and their identification is the only operation to be performed. This is of importance because different monomers are generally used together (main monomer, cross-linking agent, functionalized monomer) at different concentrations [87–90] and their individual toxicities could be very different.

Fig. 17 shows retention times from HPLC analysis of acrylic monomers used in the synthesis of DEAE-Trisacryl compared with an identical analysis of a DEAE-Trisacryl extraction solution after about 1 month at room temperature in 1 *M* sodium chloride solution.

With the same analysis, it is also possible to identify other peaks that could be of interest for toxicological studies. In this same analysis, sodium azide has been identified; its total elimination prior to protein purification is very important in order to avoid the risk of contamination of this highly toxic chemical [91].

Leakage compounds are more difficult to identify. Chemical hypotheses must be established and an intensive analytical investigation has to be done using above-described methods.

In the case of less complex packings, such as size-exclusion media, ion exchangers and hydrophobic supports, a hypothesis can be established relatively easily. Standard model molecules can then be prepared and used to prove that the hydrolysis products are effectively the same. If so, the same model molecules can be used for extensive toxicological studies.

As far as the leakage of macromolecular ligands is concerned, the problem is more complex. An immobilized protein can be released as such or in small peptides as a consequence of a proteolytic action. In this situation, it is obviously difficult if not impossible to identify each antibody fragment individually in order to carry out toxicity studies for each of them. Here a possible approach could be to quantify the hy-

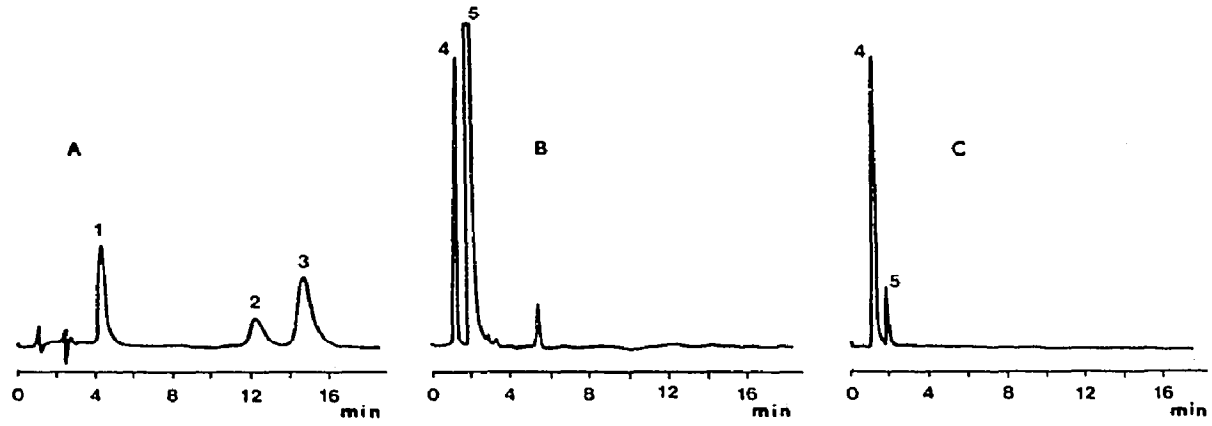

Fig. 17. HPLC used to detect and quantify acrylic monomers possibly present in DEAE-Trisacryl. (A) Chromatogram obtained with standard monomers. 1 = N-Acryloyl-2-amino-2-hydroxymethyl-1,3-propanediol; 2 = N,N'-methylenebisacrylamide; 3 = diethylaminoethylacrylamide. (B) Chromatogram of a DEAE-Trisacryl supernatant containing (4) sodium chloride and (5) sodium azide. (C) Effluent after washing the column with four volumes of sodium chloride solution. The additional peak at 5.5 min in (B) represents small traces of Triton X-100 used in the final stage of production. It is totally removed after washing with two column volumes.

drolysis phenomenon by means of polyclonal antibodies first, and whenever possible accumulate enough hydrolysis material to perform toxicity studies on the whole material.

The toxicity of well known macromolecular ligands has been reported; this is the case with lectins and to some extent protein A, all of them being classified among biologicals showing mitogenic properties [92,93].

Obviously the storage conditions of affinity media containing macromolecular ligands have a strong effect on the extent of leakage. The packing material should be stored in clean conditions to prevent any microbial growth secreting proteases that may have a rapid deleterious effect on the proteinaceous ligand. Antimicrobial agents, such as sodium azide, thimerosal, high salt concentrations and 20–40% ethanol are strongly recommended.

6.2. *Toxicity studies*

Toxicity studies of chromatographic sorbents for the separation of biomolecules have been relatively neglected in spite of the extensive utilization of this material in the industrial separation of a number of injectables. It seems obvious that if packing material is extensively washed prior to use or after a cleaning cycle and the possible released material is removed to below the sensitivity of the most selective analytical method, toxicity studies would be useless. However, chemical accidents or operator mistakes could occur any time. For this reason, toxicity studies are increasingly recommended to document processes involving chromatography to be submitted to regulatory commissions. For all possible chemical and biochemical molecules that can contaminate the protein to be purified, toxicity data generally come from in vitro assays. In-vivo assays are mostly limited to LD-50 on rats or mice or to acute toxicity only.

A number of tests exist to check the toxicity of chemicals in vitro. Culture of various animal and human cells (established lines, primary cells, hybridomas, etc.) can be used. The chemicals to be tested, in addition to the final chromatographic packings, are added at different concentrations to the culture medium and the cells are cultured in comparison with a standard culture carried out in parallel under the same conditions. Effects due to the tested biochemicals are determined by measuring the cell density, the cell growth curves, cell behaviour and morphological changes. For these studies it should be noted that several passages must be done and cultures have to be prepared in triplicate. These precautions are justified by possible delayed effects of studied chemicals that are not necessarily evidenced in the first cell generations.

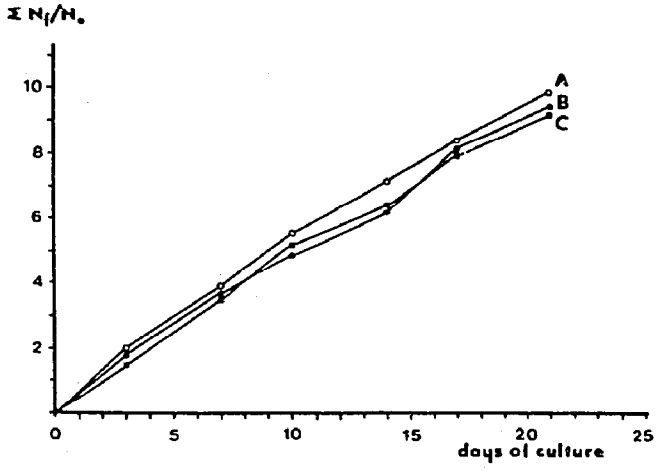

Fig. 18. Growth curves of MRC-5 human cells in vitro over 21 days in standard culture conditions in the presence of (C) 1000 ng/ml of sodium acrylamidomethylpropylsulphonate and (B) 1000 ng/ml of methacrylamidopropyltrimethylammonium chloride hydrochloride. (A) Growth curve obtained in the absence of any additive. N_f = final cell number; N_0 = initial cell number.

Moreover, cells cultured in vitro frequently change their growth behaviour, which leads to a non-homogenous cell population.

An example of cell culture as a means of detecting possible toxicity of monomers regularly used to make chromatographic sorbents is illustrated in Fig. 18. Methacrylamidopropyltrimethyl ammonium chloride hydrochloride and sodium acrylamidomethylpropylsulphonate as monomers to make strong cation- and anion-exchange resins such as Q- and S-HyperD were investigated. Both were used in the concentration range 1–1000 ng/ml in the culture medium. The growth curves clearly demonstrated that the cell behaviour was not modified in the presence of monomer even at high concentration. The shape and general aspect were also unmodified. When this experiment was extended to six culture passages over a total period of cell culture of 21–22 days, the number of cells, passage after passage, remained very close to the standard culture (see Table 7).

Another classical way to determine the toxicity of chemicals is to measure cell proliferation by the ability of cells to synthesize DNA (integration of labelled thymidine) compared with cells in normal conditions of growth. Any modifica-

Table 7
Influence of two major monomers used for ion exchanger synthesis on the growth of MRC-5 human cells in culture

Type of monomer[a]	Concentration (ng/ml)	Passage number	Number of cell generations (N_f/N_0)[b]		Relative growth (%)
			Without monomer	With monomer	
MAPTAC	100	1	2.456	3.130	127
		2	4.668	3.407	111
		4	2.487	1.588	64
		5	2.283	1.988	109
MAPTAC	1000	1	2.456	3.960	161
		2	4.668	3.513	75
		4	2.487	3.188	128
		6	2.283	2.510	110
AMPS	100	1	3.613	3.300	91
		2	3.367	3.690	110
		4	3.099	2.642	85
		6	2.942	3.639	124
AMPS	1000	1	3.613	4.312	119
		2	3.367	3.600	107
		4	3.099	2.840	92
		6	2.942	3.115	106

[a] MAPTAC = metacrylamidopropyltrimethylammonium chloride; AMPS = acrylamidomethylpropylsulphonic acid.
[b] N_f = initial number of cells; N_0 = final number of cells.

tion of the speed in DNA synthesis may be interpreted as potential toxicity of the tested molecule, confirmed or not by further investigations.

Cells cultured over six passages or more are also a good source for chromosomal analysis studies. For instance, human lung fibroblasts (MRC-5 cells ATCC No. CCL-171) have been cultured in the presence of chemicals used for chromatographic packing synthesis, and also with leached dye material [71,97]. The general approach for this study is to culture cells in the presence of a large amount of the studied chemicals (at least a tenfold concentration compared with the maximum amount that can be found within the sorbent or leached from it) and compare them with a standard culture.

MRC-5 cells taken at the 29th passage are then cultured over six passages (about 3 weeks of culture) and then mitoses stopped in prometaphase–metaphase stage using colchicine. Chromosomes are recovered after a hypotonic shock and classically stained according to a Giemsa procedure. Selection of mitoses has to be done according to their photogenic aspect in the absence of any overlapping. About 500 metaphases per slide have to be selected and the observation extended to over four–five slides. Chromosomes have to be identified, assembled and counted and polyploidia situations identified. The maximum level of polyploidia accepted is 17 over an average of 500 observed metaphases; above this number the investigated chemical is classified as toxic. A number of such determinations have been done in our laboratories with chemicals used for chromatographic sorbent synthesis, ligands and material leached from columns submitted to extreme chemical conditions. Table 8 shows polyploidia results from two major chemicals used in the preparation of ion exchangers, and also from three native dyes used as affinity ligands and a dye derivative identified as a leached molecule from a synthetic blue resin submitted to extreme alkaline conditions [70]. It can be seen that in all instances the level of induced polyploidia is below the limit of acceptance and very close to what was found with cells cultured in the absence of any chemical.

Genotoxicity studies are also of importance for free chemicals generated by chromatographic packings. Common genotoxicity determinations can be done in vitro using special recombinant *E. coli* strains. The method implies a culture of this strain in the presence of chemicals under investigation. Recombinant *E. coli* possesses a β-galactosidase gene (*LacZ*) controlled by a gene with an SOS-type function (*SfiA*) involved in the cell division [94]. In the presence of genotoxic material interacting with bacterial

Table 8
Study results of polyploidia level induced by different chemicals on MRC-5 human cells cultured over six passages (from 29th to 36th)

Chemical molecule studied[a]	Number of polyploidia cases per given amount of metaphases						
	Slide 1	Slide 2	Slide 3	Slide 4	Slide 5	Average	Average (%)
Standard without any chemical	9/513	3/514	9/511	7/507	6/507	7/509	1.37
AMPS	9/504	12/508	1/514	7/508	15/516	11/510	2.15
MAPTAC	12/511	15/523	16/517	16/505	7/509	13/513	2.53
Reactive Blue-2	17/509	12/536	5/505	8/509	9/525	10/517	1.93
Reactive Red-120	4/504	9/503	7/514	9/506	11/503	8/506	0.58
Reactive Yellow-13	5/514	9/506	5/514	7/513	9/522	7/514	1.36
Carboxy R.B.-2[b]	0/266	3/231	10/391	12/367	9/490	7/349	2.00

[a] Cells were cultured in DMEM medium supplemented with 7.5% of foetal bovine serum; initial cell concentration was 1.3×10^6 per 25 cm^2 flask.
[b] Concentration of chemicals was 1–10 μg/ml of culture medium.

DNA, the SOS system is activated; the activation is proportional to the lesion extent and induces the production of β-galactosidase. This enzyme can then be easily quantified using specific chromogenic substrates. The enzymatic activity is proportional to the genotoxic power of the investigated chemical and its concentration. Absence of β-galactosidase activity, however, cannot be considered as a sign of the lack of any genotoxic activity; investigated chemicals could in fact induce the inhibition of the total protein synthesis giving rise to false-negative results. To avoid this possible problem, normal constitutive alkaline phosphatase must be determined as a control. It must also be noted that with complex investigated molecules, a negative result cannot be considered safe in vivo because possible metabolites of these molecules could induce genotoxicity. To avoid this other potential problem, parallel studies have to be done after incubation with a liver extract fraction that is assumed to contain complex enzymatic systems capable of degrading, as in vivo, the investigated molecule with the production of metabolites [95,96]. Negative controls and positive controls with, e.g., 4-quinoline oxide or/and 2-aminoanthracene (well known genotoxic molecules) must complete these studies.

Only a few investigations have been published in connection with chromatographic materials. Our group carried out a number of investigations especially with chromatographic-related dyes because one of the major reasons why immobilized dyes are not very popular on the production scale is their unknown level of toxicity. Our studies concerning Reactive Blue-2 and Reactive Red-120 and some derivatives as possible leached materials from chromatographic separations demonstrated a total absence of genotoxicity, at least at concentrations below 40 μg/ml [71,79], which is much larger than the leakage levels ever seen when separating proteins on immobilized dyes.

Further in vitro-related studies could involve the interaction of leached material with cell membranes and consequently possible internalization into cell compartments. Localization of chemical compounds inside the cells, determined using radiolabelling or specific antibody detection, is also of importance when specific parts of the cells concentrate the target chemical, e.g., membranes, nucleus, ribosomes or DNA.

In vivo studies are not widely used to check the toxicity of chromatography-related chemical material except for the LD-50 and less frequently local tolerance tests in rabbits. Published examples of LD-50 studies again deal with dyes and derivatives on mice where amounts as large as 2 g of dye per kilogram of tissue have been administered in the food at a single dose. The mouse population was constituted of males and females in equal numbers and their behaviour and mass were followed over 8 days [98]. In this particular case no abnormal reactions were observed; all the mouse population remained alive. Statistical variations in mass were found similar to a control population with modifications as low as +2.9% for females and +7.6% for males. No modification of apparent behaviour was disclosed. As far as cutaneous irritation tests in rabbit are concerned, it can be stated that this technique is applied to any liquid, pasty, powdery and solid substrates which are layered on the skin of Albino rabbits. For very acidic substances ($pH < 2$) and very alkaline products ($pH > 11.5$), probable corrosive properties must also be taken into consideration.

Tests are generally done on about six adult rabbits selected randomly and being in apparent good health and vaccinated. Tested chemicals have to be applied to the rabbit on the naked skin using 0.5 ml of solution previously adsorbed on an eight-layer gauze pad of about 2.5 cm^2 and kept in contact for 4 h. Results obtained after 1, 24, 48 and 72 h are classified into five levels for both erythema or eschar formation and oedema formation. Interpretation of results and scoring are effected according to classically established criteria in comparison with a standard population of control rabbits.

Special studies carried out with N-acryloyl-2-amino-hydroxymethyl-1,3-propanediol and N-methacryloyl-2-amino-2-hydroxymethyl-1,3-propanediol monomers used for the preparation of Trisacryl resins and diethylaminoethylacrylamide, for instance, demonstrated their innocui-

ty at concentrations as high as 2000 mg/kg of rabbit and classified as non-toxic chemicals for this particular test. Such an amount enormously exceeds what could be found as contaminants in the presence of a resin which has been quantified in amounts below 0.5 ppm (sensitivity of the test method).

This assembly of "tools" to determine the level of toxicity and safety for chromatographic sorbents and the examples given contribute to a better knowledge of the chromatographic process of protein separation. The risk of contaminating pure biologicals with toxic material is, however, extremely low because first the concentration of leaching levels of resins when in regular use is in the worst case very low and second because of the non-toxic material generally chosen for making the resins themselves.

Finally, toxicity investigations and knowledge should be extended to chemicals used in buffers, and to impurities present in the feedstocks and that could be present in trace amounts in the final products. This is obviously a case-by-case situation where the final user is the only qualified judge to decide about the investigations to be carried out in compliance with regulatory recommendations and in connection with the sorbent supplier.

7. Future trends

In addition to the chemical structure of a protein considered for therapeutic use, the compound is still defined by an operational description. That implies that the exact protocol of the production and purification process is part of the definition of the compound. This approach provides consistency and safe production methods, but it also inhibits the rapid development of novel processes. To overcome this situation and in order to have a strategy to accelerate the development of rDNA-derived therapeutics, full insight into the process is a prerequisite. Validation of cleaning and a full understanding of how a chromatographic process behaves in long-term operation are considered as major contributions to this effort. Recent regulations, allowing the multi-purpose use of a facility for the production of a recombinant protein, also require rules and methods for cleaning validation. Sufficient knowledge of the lifetime of a sorbent has to be provided.

Recent studies have shown that for complete aseptic processing, the conventional procedures for sanitization are not satisfactory. Sorbents resistant to oxidizing agents combined with appropriate sanitization protocols must be developed. Ready-to-use preparative columns with a validated clearance of adventitious agents and a validated chemical and physical degradation behaviour may become a reality.

New chromatographic applications will be developed in DNA technology in connection with gene therapy. The amount of pure DNA fragments to be produced will increase dramatically in the future and special packings will be developed and used. The total elimination of high-molecular-mass DNA impurities tightly adsorbed on a column may be a problem that will need to be completely solved in the future. New cleaning–hydrolysing non-toxic agents have to be identified and validated in this respect. General standardization of the main chromatographic methodologies (buffers, speed, cleaning agents, etc., in relation to the nature of the sorbent) should force one to simplify the procedures that any user is developing independently.

Trade names

Bio-Gel	Bio-Rad, Richmond, CA, USA
DEAE-Sepharose Fast Flow	Pharmacia–LKB Biotechnology, Uppsala, Sweden
DEAE-Spherodex	BioSepra, Marlborough, MA, USA
DEAE-Trisacryl	BioSepra, Marlborough, MA, USA
Fractogel	Merck, Darmstadt, Germany
Phenyl-Sepharose	Pharmacia–LKB Biotechnology, Uppsala, Sweden
Poros	Perspective Biosystems, Cambridge, MA, USA

Q- and S-HyperD	BioSepra, Marlborough, MA, USA
S-Sepharose Fast Flow	Pharmacia–LKB Biotechnology, Uppsala, Sweden

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