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Review

Analysis of the *Cinchona* alkaloids by high-performance liquid chromatography and other separation techniques

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Abstract

The *Cinchona* alkaloids, which include the pharmaceuticals quinine and quinidine, continue to have a wide variety of important uses. A number of different chromatographic procedures have been developed for the qualitative and quantitative analysis of these compounds in a variety of sample matrices. Reversed-phase HPLC using ODS columns in combination with acidic mobile phases, and UV detection, is the most widely used method. Nevertheless, precautions need to be taken due to the strong silanophilic interactions which can occur with these analytes and the column surface, which can lead to poor peak shape and resolution. Different selectivity may be achieved in HPLC separations by use of alternative stationary phases, or by varying mobile phase pH. The specificity of detection systems may be improved by use of photodiode array UV detectors, or especially mass spectrometers. Thin-layer chromatography (TLC) provides a cheap alternative analytical method, which is especially useful for qualitative analysis. High-performance TLC, gas chromatography, capillary electrophoresis and capillary electrochromatography are all methods which after some development, could prove useful for *Cinchona* alkaloid separations.

Keywords: Reviews; Optimisation; Retention mechanisms; Stationary phases, LC; Alkaloids; *Cinchona* alkaloids

Contents

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1. Introduction

The *Cinchona* alkaloids are a group of about 35 bases occurring in the bark of *Cinchona* and *Remijia* species which are indigenous to the Andes but have been cultivated commercially in South and Central America, India, Sri Lanka, and the East Indies [1,2]. The principal *Cinchona* alkaloids are the quinoline alkaloids quinine, quinidine, cinchonine and cinchonidine. The ''average'' bark contains 7–12% total alkaloids, of which quinine accounts for 70–90%, cinchonidine 1–3%, and quinidine up to 1% [3]. Bark preparations have been used for the treatment of malaria for centuries, and the history of the early uses of quinine and other *Cinchona* alkaloids, together with their isolation and structural determination, have been documented recently [4]. For the last 50 years or so quinine has suffered competition from synthetic anti-malarial drugs such as chloroquine. However, resistance of the parasite *Plasmodium falciparum* to quinine appears to be significantly less than with chloroquine [5–8], thus quinine is still widely used. Other advantages of quinine are its relatively low price and wide availability [3]. Quinine and current synthetic antimalarials may suffer competition from other drugs or by the development of a vaccine. However, there are also many other uses of the *Cinchona* alkaloids. Quinine is used as a bitter flavouring in drinks and in many foodstuffs. Paracetamol tablets commonly contain quinine as a febrifuge that also imparts an unpleasant bitter taste discouraging inappropriate use. Quinidine, which can be prepared from quinine, is used as a cardiac anti-arrythmic drug and in the treatment of atrial fibrillation. About half of the world market for quinine is used for the synthesis of quinidine [3]. An important use of the principal alkaloids is their wide application as catalytic reagents in chiral organic synthesis [9,10], or to produce high-performance liquid chromatography (HPLC) stationary phases for chiral separations [11–13].

Because of the expense of synthetic methods to obtain quinine, *Cinchona* bark remains the primary source of this compound. Analytical procedures are necessary, for example, to assess the quality of *Cinchona* bark, which is harvested usually after 7–12 years of growth [2]. Despite the average values quoted above, the alkaloid content of the bark is subject to considerable variations, occasionally it can be as high as 18–25% [3,14] but in other cases, much less than the typical levels, giving barks of little commercial value. The alkaloid profile of the bark as well as the total alkaloid content affects its usefulness. More recently, attempts have been made to produce *Cinchona* alkaloids through cell cultures. This work has been driven by the long lead time between planting and harvesting of trees and the rather unpredictable plant to plant variation in alkaloid yield [3]. A considerable number of publications appeared in the 1980s detailing attempts to produce the *Cinchona* alkaloids by culture methods, and there was much interest in the analytical methods to determine alkaloids in them. However, the yields of cultures proved to be rather low. It is possible that the production of alkaloids in *Cinchona* can be encouraged by genetic modification, which may generate further analytical research on such samples. In general, both biotechnological procedures for the production of *Cinchona* alkaloids and subsequent analytical methods have focused on the quinoline alkaloids due to the established demand for quinine and quinidine. Nevertheless, some methods for analysis of the indole alkaloids have been published. These contain a five-membered rather than a six-membered heterocycle fused to the benzene ring [15]. Analytical procedures have also been developed for the assessment of quinine or quinidine and their metabolites in human biological fluids, since these drugs tend to have narrow therapeutic limits, being toxic in high dose but ineffectual if the dose is too low [16,17]. The most common method of analysis for all samples has been HPLC in the reversed-phase (RP) mode, although other HPLC separation mechanisms, principally normal-phase chromatography on silica columns, have also been used. A variety of detection systems such as UV,

fluorescence and mass spectrometry (MS) have been employed. Thus HPLC analysis will be the major topic of this review. Nevertheless, significant analytical work has been performed using thin-layer chromatography (TLC), and both gas chromatography (GC) and capillary electrophoresis/capillary electrochromatography offer at least a potential alternative to HPLC procedures. Earlier applications of these separation methods to analysis of *Cinchona* alkaloids have been reviewed by Baerheim Svendsen and Verpoorte [18]. In the present review, alternative procedures to HPLC will receive briefer comment in line with the extent of their use.

Many different HPLC procedures have been published using a variety of stationary and mobile phases. Many of these papers deal with the analysis of *Cinchona* alkaloids as a group and are orientated towards use of the method in phytochemical studies, as described above. Other papers have a more biomedical orientation and are concerned more with the pharmaceutical analysis of (particularly) quinine and quinidine and separation of the metabolites of these compounds produced in body fluids. This review will concentrate on the former application, that is the separation of the alkaloids themselves. Relatively few papers (especially in the biomedical group) give much attention to the separation mechanisms involved in the analysis of the alkaloids. Thus, rather than merely list all papers referring to analysis of the alkaloids, it is hoped to instead discuss some of the more fundamental principles of the analysis and to direct the reader to those methods which are likely to give optimum results or at least offer the possibility of distinct selectivity effects. Finally, methods for the extraction of *Cinchona* alkaloids prior to analysis will be discussed.

2. High-performance liquid chromatography

2.1. Reversed-phase separations

2.1.1. General

RP separations on bonded silica have been by far the most widely used for analysis of the *Cinchona* alkaloids, probably due to their perceived general advantages, including UV transparency and cheapness of the typical mobile phases, reproducibility of retention times, compatibility with aqueous samples and high column efficiencies. The application of RP chromatography to the separation of the alkaloids is not straightforward, however, due to their basic nature and thus possible interaction with silanol groups. Due to steric effects, a considerable number of silanol groups (as many as half [19]) can remain underivatised on bonded RP packings. End-capping procedures, where underivatised silanols are further reacted with a small silylating agent such as trimethylchlorosilane, can remove the influence of some of the more active silanols. However, the total number of reacted silanols is largely unaltered by this procedure [20]. Quinine (see Fig. 1) has two basic nitrogen atoms of pK_a approximately 4.3 and 8.5, (the quinoline nitrogen having the lower basicity) and thus the molecule is likely to be at least partially protonated over the whole pH range of operation of RP columns (pH 2–8). The underivatised column silanol groups have an average pK_a of 7.1 [19], although this average conceals a range of acidities, with some silanols likely to have a pK_a<3. Thus, ion-exchange interactions can occur between protonated alkaloid and dissociated silanol groups over a wide pH range. These, and possibly other interactions such as hydrogen bonding can give rise to tailing peaks, poor column efficiency and irreproducible retention times for a variety of quite complex reasons [20]. Tailing is extremely undesirable when separating complex mixtures of compounds due to loss of resolution, and also due to the effects it has on quantitative results. For example, integrators may not be able to establish the end of a tailing peak with sufficient precision or accuracy. Retention times may be considerably affected by sample mass (see below). Irreversible adsorption, either partial or complete, may occur for some basic compounds. Supposedly similar columns bonded with the same ligand may show completely different silanol effects due to differences in the base silica, the choice of which is discussed below. Worst results seem to be obtained on the older ‘‘classical’’ RP which are made from lower purity silica than more recent phases. It is possible that poorer performance may be attributed to their high metal content, particularly with regard to iron and aluminum which can become incorporated into the silica structure, increasing the acidity of neighbouring silanol groups

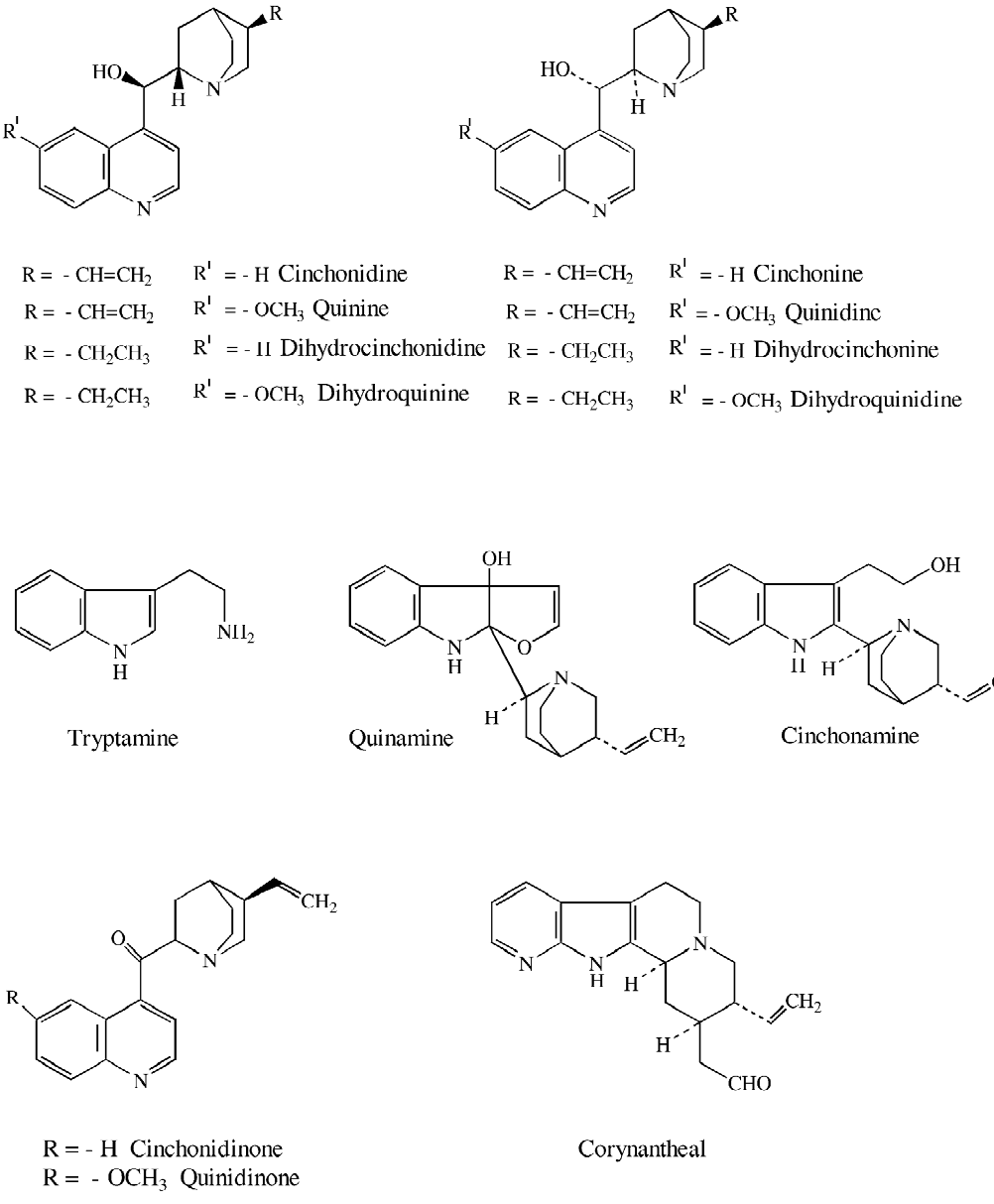

Fig. 1. Structures of some *Cinchona* alkaloids.

[21]. However, it is difficult to attribute the success or failure of a given phase to a particular feature, because phases differ in so many ways, e.g., nature of the base silica, surface area, pore size, nature of bonding reaction and ligand, endcapping, etc. It is possible to mask the effects of silanol groups by use of mobile phase additives ([22–24], see below), alternatively, phases of low silanol activity can be chosen. All silica-based columns will show silanol effects to a greater or lesser degree; different workers have used mostly octadecylsilyl (ODS) columns (C_{18}) but columns with shorter alkyl chains and cyanopropyl columns have also been used for alkaloid analysis. Most workers have used low pH

(2–3) because here the ionisation of silanol groups (apart from the most acidic) is largely suppressed, giving rise to better peak shape. However, it is possible on modern RP columns to obtain reasonable peak shapes for the alkaloids at intermediate pH (pH 7). The relative merits of these methods will be discussed below.

2.1.2. Choice of column

Table 1 lists retention factor, column efficiency (N) and asymmetry factor (A_s) for 24 columns using a pH 3 phosphate buffer modified with acetonitrile (these are recommended optimum mobile phase conditions—see below. No silanol masking agents were used). It can be seen that there is a considerable variation in the peak shape for quinine given by these different phases, ranging from extreme peak asymmetry ($A_s>5.0$) and very poor efficiency (500 plates) for Hypersil ODS to good peak asymmetry ($A_s<1.4$) and high column efficiency (15 000–20 000 plates) given by some of most recently introduced phases. While the peak shape given by a packing has been shown to be very dependent on individual features of the solute such as pK_a and stereochemistry, that of the four major alkaloids and their dihydro derivatives seems to vary relatively little amongst themselves, probably due to the similarities in their structures [25–31]. The first seven phases in Table 1 are ''classical phases'' which have been available for many years. In general, these give very poor results for the analysis of quinine (see above, [32]). Of these older phases, only μ-Bondapak C_{18} gives reasonable peak shape. LiChrosorb RP 8 Select B was amongst the first designed especially for the analysis of basic compounds, and can be seen to give improved results for quinine compared with the ''classical'' phases (Table 1, Fig. 2). The rest of the columns listed are considered as

Table 1
Retention factor, column efficiency and asymmetry factor for quinine on phases using acetonitrile–0.0265 *M* phosphate buffer, pH 3.0 (15:85, v/v)

Column	L×I.D. (cm)	d_p	Surface area (m^2/g)	% C	k	N	N(df)	A_s
μ-Bondapak C_{18}*	30×0.39	10	330	10	4.3	4500	–	1.8
Novapak C_{18}*	15×0.39	4	120	7	8.4	4300	–	2.9
Hypersil C_{18}*	25×0.46	5	170	10	6.7	500	–	>5.0
Spherisorb ODS-1*	25×0.46	5	200	7	12.0	2200	–	3.1
Spherisorb ODS-2*	25×0.46	5	200	12	3.1	7200	–	2.7
LiChrosorb RP-18*	25×0.46	5	300	16	4.3	1280	–	4.0
Nucleosil 5 ODS*	25×0.46	5	100	6	2.2	6200	–	2.3
LiChrosorb RP8 Select B*	25×0.46	5	500	11.5	8.3	11 000	–	1.4
Inertsil ODS	25×0.46	5	350	18.5	7.3	14 200	12 800	1.0
Inertsil ODS-2	25×0.46	5	320	15	3.6	16 700	13 800	1.3
Inertsil ODS-3	25×0.46	5	450	15	7.0	13 800	11 400	1.4
Inertsil C8-3	25×0.46	5	450	9	5.6	13 700	11 800	1.2
Inertsil cyanopropyl-3	25×0.46	5	450	14	0.2	8210	6230	1.3
Kromasil C_{18}	25×0.46	5	340	19	5.6	14 200	10 300	1.4
Kromasil C_8	25×0.46	5	340	12	5.5	16 300	12 600	1.4
Symmetry C_{18}	25×0.46	5	330	19.5	5.2	11 100	5130	2.3
SymmetryShield RP-18	25×0.46	5	340	17.5	2.2	13 500	12 300	1.2
SymmetryShield C_8	25×0.46	5	340	15	3.3	14 600	12 600	1.2
Supelco ABZ+	25×0.46	5	170	12	1.4	10 500	7780	1.4
Supelco Discovery C_{18}	25×0.46	5	200	13.5	2.4	13 800	11 500	1.3
Supelco Discovery Amide	25×0.46	5	200	12	0.8	19 100	16 500	1.2
Supelco Discovery C_8	25×0.46	5	200	7.5	2.7	16 800	14 600	1.2
Purospher	25×0.46	5	500	18.5	0.9	4200	2200	2.0
Luna C_{18} (2)	25×0.46	5	410	17.5	4.9	18 500	13 900	1.5

*=Used 0.1 *M* phosphate buffer. Results would be expected to be slightly worse for these columns using 0.0265 *M* phosphate buffer. All columns 5 μm particle size except where stated otherwise.
N=Column efficiency measured at half-height. N(df)=column efficiency measured according to the Dorsey–Foley procedure [29] which gives a better indication of true column efficiency.

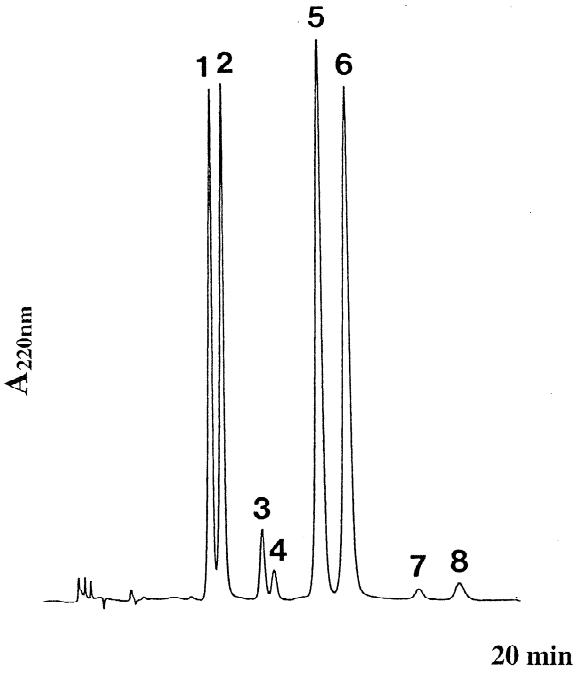

Fig. 2. HPLC separation of alkaloids on the LiChrosorb RP-8 Select B column. Peaks: 1=cinchonine, 2=cinchonidine, 3=dihydrocinchonine, 4=dihydrocinchonidine, 5=quinidine, 6=quinine, 7=diydroquinidine, 8=dihydroquinine, Detection, UV at 220 nm. Flow-rate 1 ml min^{-1}. Eluent, 15% acetonitrile in 0.1 *M* phosphate buffer adjusted to pH 3.0 with phosphoric acid before modifier addition (see Ref. [23]).

''new generation'' reversed phases which are based on pure silica and/or designed especially for basic compound separation; many give some further improvement in the peak shape for quinine compared with LiChrosorb RP-8 Select B. For some phases based on the same silica, incorporation of an embedded polar group in the alkyl chain is seen to improve performance (compare for example the performance of Symmetry C_{18} with SymmetryShield RP18, containing an embedded carbamate group. A smaller improvement is shown for Discovery Amide, containing an embedded amide functionality, compared with Discovery C_{18} [27]). It is possible that phases with an embedded polar group can trap a deactivating layer of water close to the silica surface, thus giving some shielding of the silanol groups. Alternatively, there may be some deactivating effect of hydrogen bonding between the polar group and the silica surface. In general for basic compounds, shorter alkyl chain C_8 phases have been found to give better results than C_{18} phases on the same silica [27]. However, at least for quinine using the mobile phase shown (Table 1), these advantages seem to be slight (compare Discovery C_{18}/C_8, Kromasil C_{18}/C_8, Inertsil 3 ODS and C8-3). Table 1 is slightly misleading in that it contains a high proportion of ''new generation phases''. There are many classical phases still in common use (although not listed) which would be expected to give very poor results for the analysis of *Cinchona* alkaloids. Table 1 should be treated with some caution. It is possible that manufacturers continually improve some named phases. Results obtained for some phases several years ago may give an unduly pessimistic view of the performance of the same phase purchased more recently.

While Table 1 is a guide to peak shape, it is not an infallible guide to resolution of the alkaloids, since this is also affected by their relative retention. Retention of basic compounds in RP chromatography is an extreme complex process which is not completely understood, and a variety of factors are likely to contribute to the resolution of a mixture of such compounds. The relative magnitude of each of the retention processes may also vary from column to column, affecting the resolution of different compounds. LiChrosorb RP-8 B has been shown to give baseline resolution of the eight major alkaloids using the mobile phase conditions shown. However, resolution of the four major alkaloids and their dihydro derivatives has not been studied in detail for all of these phases. In addition, resolution of alkaloids from matrix components in a particular sample must be considered, or indeed from minor alkaloids such as are present in *Cinchona* bark [33]. Further work on sample preparation techniques, or the use of mass spectrometric detection in conjunction with HPLC, is necessary to allow checks on chromatographic peak purity or allow selective detection of a compound in the presence of a co-eluting impurity. Alternatively, the selectivity of separations may be usefully manipulated by change of column or mobile phase conditions (see below).

LiChrospher RP-Select B (a spherical version of the LiChrosorb phase) was also used by Nielsen et al. for the separation of quinine and its two major

metabolites, (3S)-3-hydroxyquinidine and quinidine oxide in plasma and urine [16].

A number of alternative RP columns were evaluated specifically for separation of *Cinchona* alkaloids [34], particularly with a view to developing LC–MS methods (i.e., without use of buffers or tail reducing additives incompatible with MS). The columns used included a phenyl bonded silica phase, a mixed mode C_{18}-cation-exchange packing, a column based on alumina rather than silica, and a purely polymeric column. Due to different mobile phase conditions, it is difficult to compare results with those above. Also, it was uncertain whether all of the silica-based materials chosen were indeed of the type especially suitable for basic compounds (i.e., based on pure silica). However, it was possible to conclude at least that separations on the polymer column showed disappointing efficiency which is a general characteristic of this type of column. Furthermore, the alumina based column showed poor selectivity of separation. At the present time, therefore, it would appear that silica-based columns are still the first choice for RP separations of the *Cinchona* alkaloids.

Changing the chemical nature of the ligand in bonded silica RP columns from the normal C_{18} or C_8 phases may be a useful tool to alter selectivity. A separation at pH 7 was reported on a cyano column based on a traditional silica (Spherisorb CN) in the RP mode [35] with phosphate buffer, pH 7 modified with mixtures of acetonitrile, methanol and tetrahydrofuran (THF). The order of elution of the major alkaloids was quinine, quinidine, cinchonidine and cinchonine—this is different from that on C_{18} columns operated at acidic pH (see Fig. 2). It is unclear whether the different selectivity should be attributed to the cyano ligand or to the different pH of operation. However, the fact that reasonable peak shapes were obtained even on this impure silica at pH 7 is of interest. Recently, cyano columns based on much purer silicas have become available (e.g., Inertsil-3 CN [27]). There are indications of considerable changes in selectivity for general pharmaceutical compounds on this new cyano phase, although whether it will be useful for *Cinchona* alkaloid separations remains to be studied.

2.1.3. Choice of mobile phase conditions

The nature of the organic modifier can give a considerable influence on peak shape of basic compounds. In one study, eight ''new generation'' RP columns were compared for the analysis of a variety of different bases, including quinine, using acetonitrile, methanol and tetrahydrofuran at both low and intermediate pH [28,29]. However, it appeared that at least at pH 3, the peak shape of quinine is not much influenced by the nature of the modifier. The plate count (measured at half-height) and asymmetry factor of quinine averaged over the eight columns studied was 9200 and 1.5 in methanol modified, pH 3 buffer, 10 000 and 1.4 with THF as modifier and 12 600 and 1.5 with acetonitrile. Due to the higher plate count obtained, acetonitrile is probably the first choice modifier at acidic pH; indeed this result was found for many other pharmaceutical bases studied. Despite the general recommendation of acetonitrile as modifier at acidic pH, it is possible that for the separation of complex mixtures of alkaloids, useful selectivity effects may be obtained by use of the alternative modifiers. Furthermore, despite the general recommendation for acetonitrile at acidic pH, use of THF may be advantageous on columns which have higher inherent activity towards basic compounds [28,29].

Mobile phase pH is another important consideration, both in terms of optimising peak shape and altering the selectivity of the separation. pH 3 (generally accepted as the most acidic pH for long-term stability of silica-based columns) generally gives better peak shapes for basic compounds than pH 7 (accepted as the general high pH stability limit, although silica-based columns claiming stability at considerably higher pH are available). At pH 3, most silanol groups are probably undissociated even though alkaloids are protonated, thus there is limited possibility for ion-exchange effects [28,29].

However, acceptable peak shapes may be obtained on the newer RP columns even at pH 7, and use of this higher pH may offer useful selectivity effects, as has been shown clearly for the tobacco alkaloids, which elute in a different order at pH 3 and pH 7 [36]. Preliminary results also indicate this is true for *Cinchona* alkaloid separations [25]. Column efficiency and asymmetry factors have been recorded for quinine at pH 7 for eight new generation RP columns using phosphate buffers modified with acetonitrile, methanol and THF [28]. With appro-

priate modifier and column selection, efficiencies higher than 40 000 plates m^{-1} with asymmetry factor <2 can be obtained. A recent study on the latest columns [27] showed that for a given column, peak shapes at pH 7 for quinine were often comparable with those obtained at pH 3. For example 17 000 plates with asymmetry factor 1.6 were obtained for quinine on a Discovery C_8 column using acetonitrile–phosphate buffer, pH 7 which compares well with the result for acetonitrile–phosphate buffer, pH 3 (16 800 plates, asymmetry factor 1.2—see Table 1). A further possibility is to use a pH well above the alkaloid pK_a such that the solute is non-protonated even though silanols are dissociated. Some results at pH 11 have been reported for quinine although special phases which are stable at this pH are required [27]. Further studies on the effect of pH on the selectivity and ruggedness of separations are necessary. For example, although pH 3 seems to give good peak shapes on many columns (Table 1), this value is close to the second pK_a of the alkaloids, especially when considering that the presence of organic solvent may alter both pH and analyte pK_a. Working at a mobile phase pH close to the analyte pK_a may mean that small pH changes which occur between different batches of buffer could cause significant change in the degree of protonation, and thus retention factors of the alkaloids. Many of the newer RP columns are claimed by their manufacturers to be stable at pH values of 2 or even lower which may give more rugged separations than at pH 3.

No systematic comparisons of different buffer salts seem to have been carried out specifically for *Cinchona* alkaloid analysis. In general for basic compounds, potassium seems to be a better choice of buffer cation than sodium, due to peak shape effects caused by the relative strength of ion-exchange interactions [20]; most work has been carried out with potassium phosphate buffers. Addition of stronger cation exchangers such as barium ions to the mobile phase may be beneficial. Ammonium acetate was used as the buffer salt by one group [34] due to its volatility and thus compatibility with mass spectrometry. pH was adjusted with ammonia or acetic acid over a pH range 3.5 to 7.5. Some caution is necessary in that this buffer has a poor capacity over some of the stated range. However, it was reported that retention decreased and peak shape improved in the acid range, in accord with the arguments above. Increased concentrations of ammonium acetate improved peak shape, presumably due to competition for ion-exchange sites. The effect of buffer concentration was similar to that reported previously with a potassium phosphate buffer [23]. However, it should be noted in general that overall buffer concentrations (at least with inorganic buffers) above 0.1 *M* may cause problems of precipitation in combination with organic solvents, or may contribute to pump maintenance problems. It can be seen from Table 1 that an overall buffer concentration of about 0.02 *M* is entirely adequate to give good peak shapes with many new generation RP columns at acidic pH; this concentration is also suitable to maintain pH stability [20]. Despite the absence of a formal comparison it does appear that using the same column (LiChrospher RP Select B), phosphate buffers at low pH give better separations than with volatile buffers such as ammonium acetate [26,34].

Isocratic analysis appears to be satisfactory for the separation of the four major alkaloids and their dihydro derivatives [26]. However, for simultaneous analysis of the more strongly retained indole, and other minor alkaloids, gradient elution may be necessary. A good separation of 19 compounds, including the major quinoline alkaloids, together with minor compounds and possible biosynthetic precursors was reported by Giroud et al. [37], and is shown in Fig. 3. A Hypersil ODS column together with a mobile phase gradient of increasing acetonitrile concentration in ammonium formate–formic acid buffer was utilised, these buffer constituents being compatible with mass spectrometric detection. It was reported that the use of ammonium formate improved the separation of cinchonine and cinchonidine compared to ammonium acetate, another commonly used volatile buffer [38,39]. The alkaloids were eluted in three distinct groups with the simple indoles serotonin, tryptamine and 5-methoxytryptamine first, followed by the quinoline alkaloids and their dihydro derivatives, the terpenoid indoles quinamine, corynantheal, strictosidine and cinchonamine next, and lastly the cinchophyllines and 2-keto quinolines (cinchonidinone and quinidinone). The ketoquinolines gave broad peaks which was attributed to their keto–enol tautomerism.

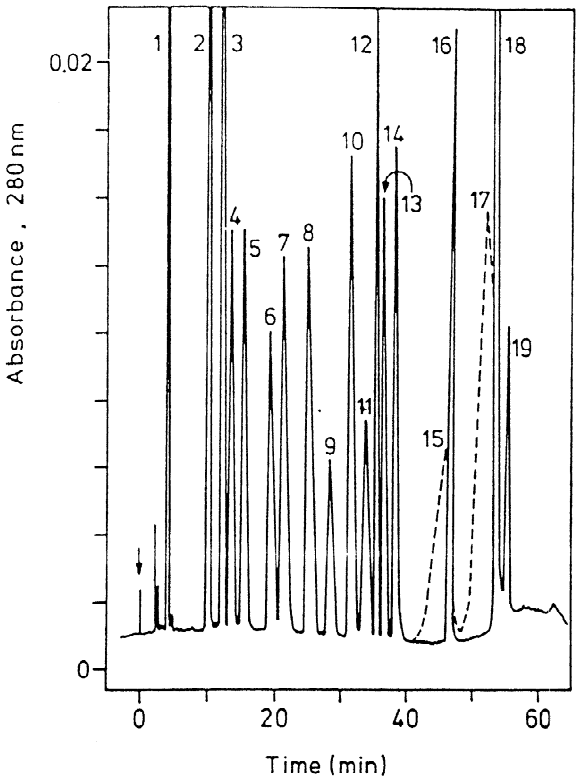

Fig. 3. HPLC separation (UV detection, 280 nm) of simple indoles, quinolines and indole terpenoid alkaloids. Column, μ-Bondapak C_{18}. Mobile phase: gradient from 100 m*M* ammonium formate–formic acid–acetonitrile, solvent A (88:4:8, v/v) to solvent B (64:4:32, v/v) in 1 h [37]. 1=Serotonin, 2=tryptamine, 3=5-methoxytryptamine,4=cinchonine, 5=cinchonidine, 6=dihydrocinchonine, 7=quinidine, 8=quinine, 9=dihydroquinidine, 10=dihydroquinine, 11=quinamine, 12=corynantheal, 13=corynantheol, 14=dihydrocorynantheal, 15=cinchoni(di)none, 16=strictosidine, 17=quini(di)none, 18=cinchonamine, 19=unknown (dashed line: separate injection of the 2-ketoquinolines 15+17).

2.1.4. Use of competitive amines to mask silanol effects

For clarity, this topic is given a separate section, although it can be regarded as an extension to Section 2.1.3.

Successful analysis of the *Cinchona* alkaloids can be obtained even on classical RP-HPLC silicas by incorporation of amines into the mobile phase which compete with the analytes for column silanol sites. For example, Hypersil ODS, was used [22] to give a complete separation of the four major *Cinchona* alkaloids and their corresponding dihydro derivatives using a pH 3 buffer containing 0.05 *M* hexylamine, modified with acetonitrile (see Fig. 4). In comparison, peak shapes on the same column without addition of the masking agent were very poor (Table 1), and some alkaloids were shown to be irreversibly adsorbed. This same classical phase was used successfully for the analysis of quinine and its major metabolite, 3-hydroxyquinine in human plasma and urine using an acetonitrile–phosphate buffer, pH 2.1 containing tetrabutylammonium bromide [40].

In another study using a Novapak C_{18} column, which is again a silica which has been available for many years, the asymmetry factor of quinidine and quinidine could be improved from about 3.0 to 1.0 by incorporation of hexylamine in a mobile phase buffered at pH 3 [23]. It appeared that longer chain amines such as hexylamine were more efficacious at silanol masking than shorter-chain compounds such as triethylamine. This may merely be due to the greater hydrophobicity of the former and thus consequently greater adsorption of the masking agent on to

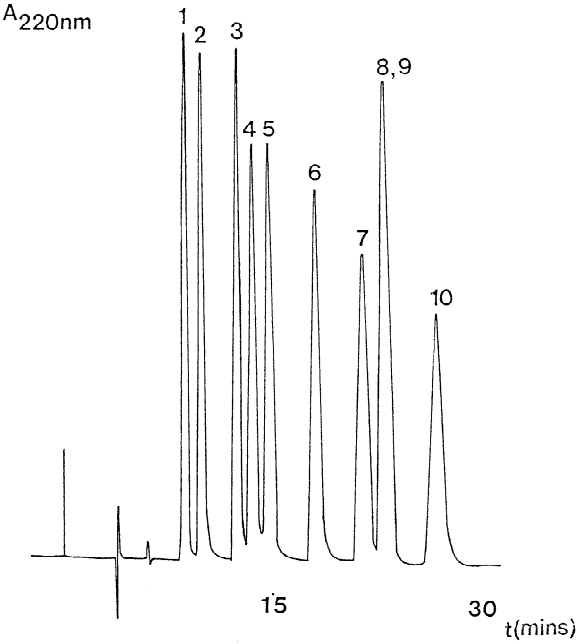

Fig. 4. HPLC separation of alkaloids on the Hypersil 5 μm ODS column. Peak identities as in Fig. 1; 9=epiquinidine, 10=epiquinine. Mobile phase 5.6% acetonitrile in 0.1 *M* phosphate buffer, pH 3.0 containing 0.05 *M* hexylamine at 1 ml min^{-1} (see Ref. [22]).

the column surface. Thus, it is not entirely clear whether a higher concentration of triethylamine will give an equivalent effect to hexylamine. Some work on elucidating the efficacy of different masking agents is still being carried out [24]. Amines with chains longer than hexylamine may give solubility problems in mobile phases with low organic modifier content, which are necessary at acidic pH to achieve reasonable k for protonated alkaloids [25]. The use of silanol masking agents can however, give rise to some difficulties. For some stationary phases, the addition of amines fails to give significant improvement in peak shape. Another problem is that column equilibration is slow and some masking agents may be difficult to remove when changing mobile phases, i.e., they may permanently alter stationary phase characteristics. Furthermore such additives contribute to the complexity of the method, and can affect detector background signals, which may be important if for example, mass spectrometric detection is used. Finally it is possible that the masking agent can chemically react with some alkaloids. For example, it was proposed that corynantheal, a precursor in the biosynthetic route of the major alkaloids which contains a free aldehyde group, undergoes Schiff's base formation with hexylamine [26]. The use of modern RP packings based on pure silica in conjunction with low pH mobile phases in any case largely obviates the need for use of these additives.

2.1.5. Effect of sample size

To obtain the best separations of complex mixtures, it is important that the stationary phase is not overloaded either in terms of sample volume or sample mass [41]. The volume or mass of sample which produces overload depends on (amongst other factors) the diameter of the column and the retention factor of the solute. Volume overload effects are likely to decrease with increasing k since the injected volume becomes small compared with the peak volume of the analyte (the volume of mobile phase which contains the analyte molecules). It is unlikely that sample volumes of 10 μl or less will cause any problem on standard bore HPLC columns, although much smaller samples may be required for microbore columns. Alternatively, mass overload of the stationary phase is likely to increase with k since a greater proportion of the injected sample is associated with the stationary phase when k is large. It was shown that as little as 10 μg of injected quinine can cause a 50% loss of column efficiency using methanol–phosphate buffer, pH 3 (65:35, v/v) on a phase which gave $k=6.4$ for quinine [42], Fig. 5. As a general recommendation for bases, some loss of efficiency and resolution may be experienced if the amount injected at acidic pH exceeds 0.5 μg using acidic mobile phases. However, for the specific case of quinine it appears that under some circumstances, this figure may be over cautious [42]. The effect of sample load on peak shape at pH 7 appears to be more complex. Overload seems to take place much less readily (possibly due to the higher number of dissociated silanol groups which exist at pH 7 compared with pH 3, although other factors may be involved). Indeed on some more active packings, the peak shape of quinine appears to improve somewhat as sample load is increased from 0.1 to 20 μg. Nevertheless, there are still problems on such packings at pH 7. The retention time of quinine (and presumably other alkaloids) can decrease significantly as the sample load increases, as it does at acidic pH. This could lead to wrong assignment of peak identity if retention time is the sole measure used for identification. It is important therefore, to match the concentration of alkaloids in samples and standards. The effects of overloading may be difficult to cope with, for instance when *Cinchona* bark is analysed which may contain large variations in the concentration of the different alkaloids. In case of doubt, samples should be spiked with standard alkaloids as an aid to peak assignment.

2.2. Normal-phase separations

Although the great majority of separations of the *Cinchona* alkaloids employ RP separations, some quite reasonable results have been published using normal-phase (NP) chromatography, mainly using bare silica columns. Indeed, an acceptable separation on silica of the four major alkaloids was published more than 25 years ago in the early days of HPLC [43]. The selectivity of the separations may be different from that in RP chromatography, offering an alternative for, e.g., resolution of minor alkaloids. In addition, peak shapes can be surprisingly good—it was recognised as early as 1982 that basic com-

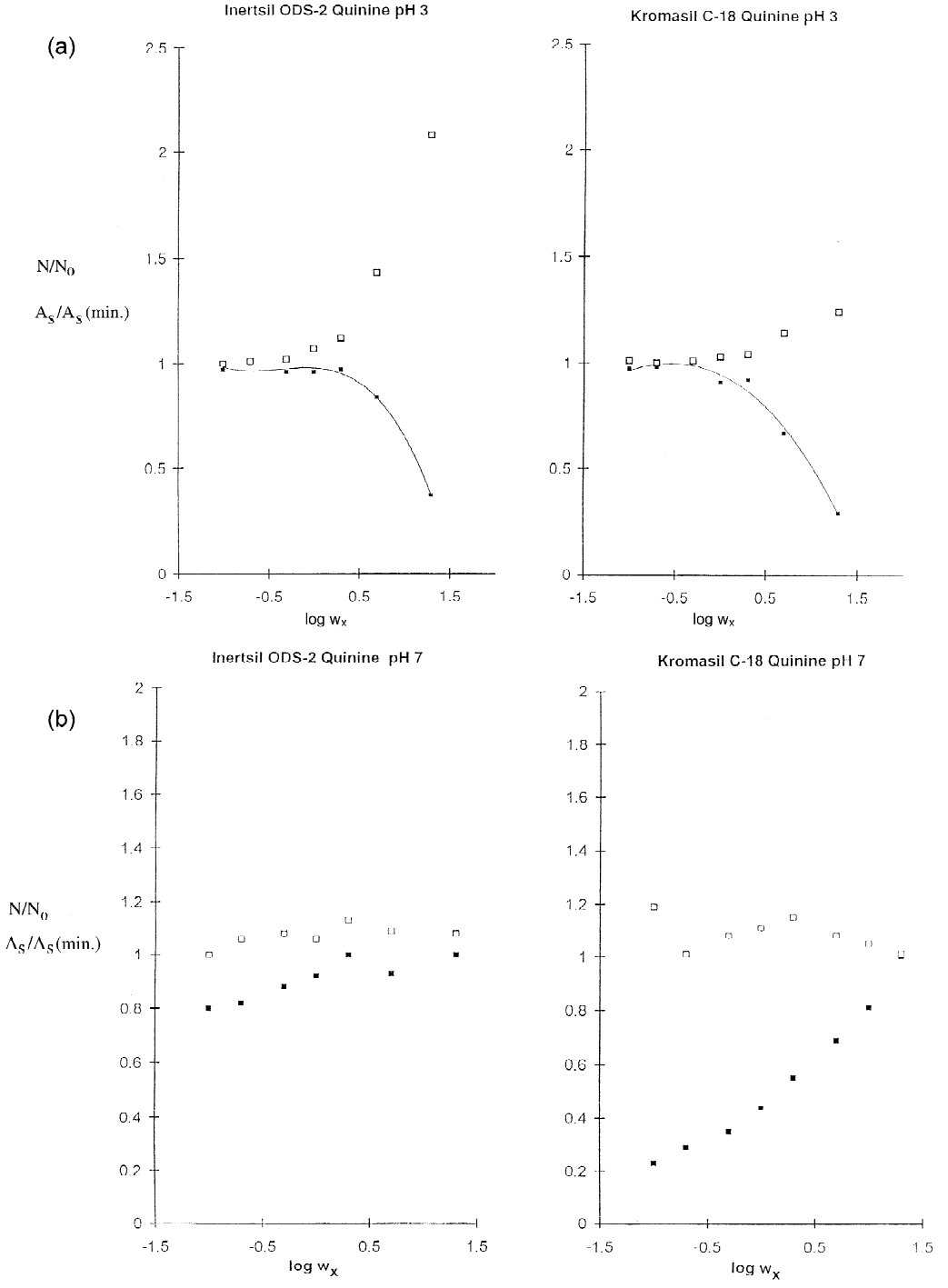

Fig. 5. (a) Plot of N/N_0, (column efficiency divided by maximum column efficiency) (black squares) and A_s/A_s (min), (asymmetry factor divided by minimum asymmetry factor) (white squares) against log (sample mass, μg) for quinine on two different ODS columns using methanol–0.0321 *M* phosphate buffer, pH 3.0 (30:70, v/v). (b) Plot of N/N_0 (black squares) and A_s/A_s (min) (white squares) against log (sample mass, μg) for quinine on two different columns using methanol–0.064 *M* phosphate buffer, pH 7.0 (65:35, v/v) (see Ref. [42]).

pounds can give better column efficiencies on lightly-loaded or bare silica than on conventional reversed-phase materials based on the same silica [44]. The reason for this observation is unclear, although may be partially due to a ''purer retention'' mechanism (mostly cation-exchange on naked silica columns as opposed to cationic/hydrophobic interactions on RP columns) or to reduced overloading effects on the larger population of silanols of a naked silica phase. It should be recognised however, that these comparative studies were performed on less pure silicas [44] available at the time. The peak shapes obtained for the alkaloids on ''new generation'' reversed phases can be very good, and this apparent advantage may no longer be significant.

Nevertheless, some separations on classical bare silicas obtained many years ago still stand comparison with more recent separations on RP packings [45,46], Fig. 6. As with TLC (see below) the mobile phases consist of non-polar solvents modified with

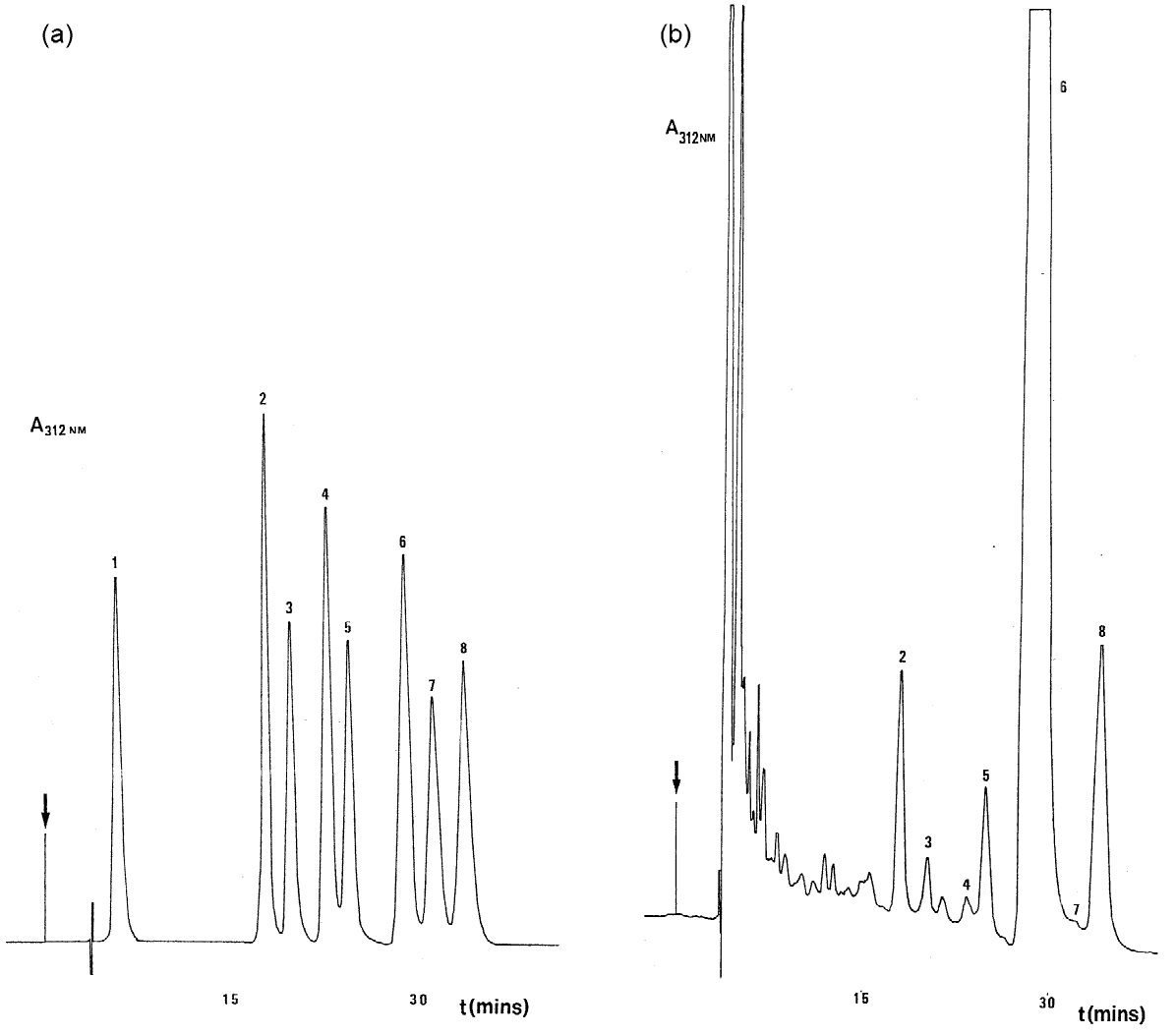

Fig. 6. (a) HPLC chromatogram of alkaloids on Hypersil silica column. Mobile phase: hexane–dichloromethane–methanol–diethylamine (66:31:2.6:0.4, v/v) at 1 ml min^{-1}. Peaks: 1=quinidinone, 2=quinidine, 3=cinchonine, 4=dihydroquinidine, 5=cinchonidine, 6=quinine, 7=dihydrocinchonidine, 8=dihydroquinine. (b) Analysis of minor alkaloids in an extract of *Cinchona ledgeriana* bark. Column and other conditions as in (a) (see Ref. [46]).

polar alcohols to adjust retention time and organic amines as tail reducing agents. In the first method [45], chloroform–isopropanol–diethylamine–water was used as the mobile phase, and in the second hexane – dichloromethane – methanol – diethylamine [46]. In both procedures, baseline separation of the four principal alkaloids was reported, and the elution order in each case was quinidine, cinchonine, cinchonidine and quinine, whereas the elution order on reversed phases at acidic pH is generally cinchonine, cinchonidine, quinidine and quinine. Thus, for complex separations it might be possible to fractionate a mixture by for example, normal-phase chromatography and chromatograph the different fractions further by reversed-phase chromatography to achieve a difficult separation.

It was recommended [46] that to achieve good reproducibility in normal-phase separations on silica the following precautions should be observed.

(i) Water should not be used as a polar modifier because it is difficult to prepare eluents with reproducible water content—methanol appears to be a good alternative polar modifier.

(ii) The column should be carefully thermostatted. Retention of the alkaloids (somewhat surprisingly) was shown to increase with increasing temperature, and significant changes in the retention factor of the alkaloids were obtained even over fairly narrow temperature range corresponding to possible fluctuations in room temperature. It is possible that as the temperature is raised, polar modifying agents are desorbed from the surface giving increasing exposure of the surface functionality [46,47]. An additional factor may be that pK_a changes of the solute occur with temperature, although study of these phenomena are complicated by the presence of the organic solvent. Note however, that it is also advisable to thermostat the column in RP methods [25], so this factor should not be considered a disadvantage of NP methods.

(iii) As with reversed-phase chromatography, the sample mass can influence the retention times. A small continuous decrease in retention was noted as sample size was increased from 20 ng to 4 μg. Again for analyses where retention time is the principal means of qualitative analysis, it is important to use standards which have concentrations reasonably well matched to those of the samples. Using these precautions, it was shown that the relative standard deviation of the retention times of alkaloid standards injected over a 12 h period was less than 0.5%, which is probably hardly worse than that obtainable even with the most modern reversed phases.

One proposed method involved a rather esoteric combination of bare silica and ion-exchange columns [48]. However, this method appears to show no particular advantage over the single silica column procedure and indeed resulted in a considerably increased analysis time.

Despite the quality of results obtainable using normal-phase chromatography, the method suffers from a number of disadvantages. These include:

(a) Non-compatibility of mobile phases (largely non-aqueous) with injection of aqueous extracts. (Nevertheless, it is relatively easy to extract the alkaloids into suitable organic solvents).

(b) Problems with gradient elution due to slow column equilibration times.

(c) Irreproducible retention times if the above precautions are not observed. Note that difficulty in control of the water content of the mobile phase may be a major source of irreproducibility.

(d) Environmental costs/impact in the disposal and use of typical NP solvents.

Simply due to the general dominance of the RP mechanism in all HPLC separations it seems likely that normal-phase chromatography will remain a minority technique for this application.

2.3. Detection of alkaloids in HPLC analysis

Most authors have used UV detection for alkaloid analysis. There is no doubt that the scope of UV detection can be considerably improved by the use of photodiode array instruments, which are capable of generating complete analyte spectra as well as conventional HPLC peaks. The originally poor sensitivity of photodiode array detectors compared with conventional variable wavelength detectors has been overcome to a considerable extent by instrument manufacturers. A problem is the generally broad nature of UV spectra which makes unequivocal qualitative identification problematic. Another difficulty is the change in the spectra which can occur for these ionogenic compounds with pH. Furthermore the spectra of the pairs quinine/quinidine and cin-

chonine/cinchonidine are identical, although these pairs of compounds are not difficult to separate by optimised RP-HPLC procedures [26]. Fluorescence detection gives much higher sensitivity for quinine and quinidine, but the low fluorescence or absence of fluorescence of many other alkaloids has limited the use of this technique. However, in biomedical studies which involve analysis of quinine and quinidine and their metabolic products in body fluids, fluorescence is a popular method [16,17,40]. MS is the method of choice for the definitive identification of minor alkaloids, and indeed for confirmation of the identity of even the major alkaloids when examining low levels in cell suspension cultures. Giroud et al. [37] used a thermospray MS interface as well as conventional UV detection at 280 nm to investigate alkaloid production in *Cinchona* shoot and compact globular structure cultures. All the alkaloids gave intense quasi-molecular ions $(M-H)^+$ but little fragmentation, an observation made also by previous workers [38]. This is disadvantageous for structural elucidation of complete unknowns, but when standards are available, the combination of authentic retention time and correct molecular ion is nevertheless a powerful confirmation of peak identity. Some compounds gave acetonitrile adducts $(M-H-ACN)^+$. The presence of most of the standard alkaloids was confirmed in the cell suspension cultures. HPLC with photodiode array detection was reported as an efficient and complementary method. The analysis of alkaloids in general using LC–MS techniques was later reviewed by the same group [49] and covered more recent interfaces such as electrospray and ionspray; however, no specific applications of these newer techniques to the determination of the *Cinchona* alkaloids was reported.

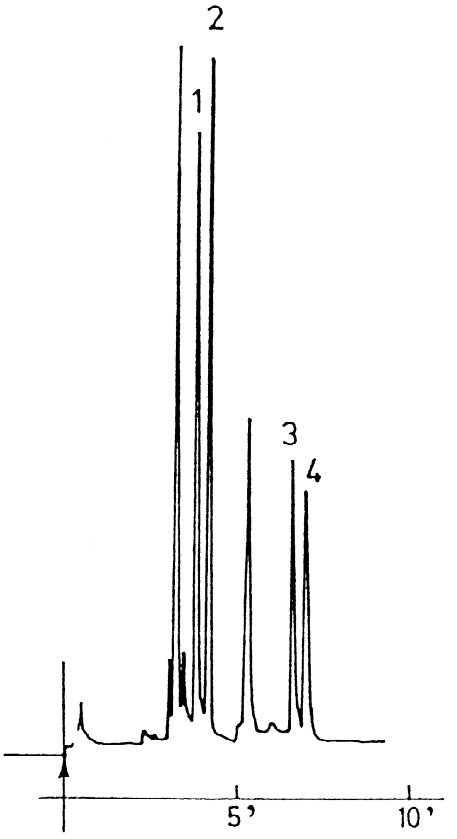

Fig. 7. Gas chromatogram of 1=cinchonine, 2=cinchonidine, 3=quinidine, 4=quinine. Column 30 m glass capillary coated with RSL-903. Temperature, isothermal 280 °C. Carrier gas, hydrogen at 5 ml min^{-1} (see Ref. [50]).

3. Gas chromatography

Verzele et al. [50] reported the separation of the four major quinoline alkaloids as underivatised compounds on an etched soft glass capillary as early as 1980 (see Fig. 7). Good peak shapes were obtained on a range of phases from non-polar to polar. However, the resolution of quinine and quinidine was zero on a non-polar phase, but increased with the polarity of the stationary phase. Baseline resolution was reported on RSL-903, the most polar stationary phase investigated, with the major alkaloids eluted in the order cinchonine, cinchonidine, quinidine and quinine. By coincidence, this is the same elution order as obtained usually with RP-HPLC at acidic pH, although it is virtually certain that different selectivities would be obtained for minor alkaloids, due to large differences in the separation mechanism of these techniques. The compounds were injected using a moving-needle injector; the column was a glass tube coated with sodium chloride dendrites prior to stationary phase coating. A different group reported that GC with mass spectrometric detection was less successful than HPLC–MS [37]; the quinoline isomers and their dihydro derivatives were not separated, and it ap-

pears that many alkaloids were not eluted. The method was based on silylation of the compounds rather than gas chromatography of the free compounds as reported previously [50], followed by analysis on a thin film non-polar column. It would appear that the use of GC for analysis of *Cinchona* alkaloids warrants further investigation to explore the apparent differences in the findings of these two groups of workers. It would be expected that modern techniques such as cold on-column injection and the use of highly inert capillary columns constructed of fused-silica and coated with polar phases might be expected to yield interesting results. A continuing difficulty with GC is the genuine and also perceived difficulties in the direct injection of aqueous solutions. However, aqueous injection with GC is possible if precautions are observed [51]. Alternatively, alkaloids could be extracted or transferred into organic solvents, obviating the need for aqueous injection. The greater general availability of GC–MS systems in comparison with HPLC–MS and the greater structural information which may be obtained from conventional electron impact (EI) spectra are both reasons for further research into GC techniques. Higher column efficiency of GC is another potential advantage, although this is counteracted by the ease of manipulation of the selectivity of an HPLC separation by adjusting the composition of the mobile phase.

4. Thin-layer chromatography

TLC has fallen somewhat out of popularity in recent years. However, until about 20 years ago, it was probably the most common method for analysis of the *Cinchona* alkaloids. TLC methods have been extensively reviewed [18,52,53]. They still retain a number of important general advantages over HPLC such as simultaneous rather than sequential sample analysis, and simple and inexpensive equipment. Perhaps a more significant advantage is the availability of specific spray reagents giving definitive colours with the different alkaloids which can act as a confirmation of qualitative analysis based on retardation factors. In contrast, for HPLC the commonly used UV absorption methods are relatively non-selective between alkaloids and matrix constituents, and spectra for alkaloids may be non-definitive (see above). It is also possible in TLC to visualise alkaloids which remain at the origin and have not moved with the solvent system. Alternatively, complete retention of alkaloids when it occurs in an HPLC column is not so easily recognised, unless analysis for that particular compound is being carried out and standards are available. Another advantage of TLC for qualitative analysis is that two dimensional chromatography can be carried out to achieve further separations of spots poorly resolved from the first run. Pound and Sears [43] used acetone–water–25% ammonia (80:20:1) and benzene–diethylamine (1:1) for the separation of the parent alkaloids and the dihydro bases of quinine and quinidine. Nevertheless, it should be noted that this separation is easily carried out by (one dimensional) HPLC due to the greater efficiency of the latter technique. Most published methods have used normal-phase TLC, using mobile phases containing solvents such as chloroform, acetone, and ethyl acetate to which alcohols such as methanol, propanol and butanol are added as polarity adjusters, with diethylamine or ammonia used to reduce spot ‘‘tailing’’. The normal-phase HPLC methods, which use similar solvent systems, were clearly developed using TLC as a guide [53].

Many interesting applications of TLC to the analysis of the *Cinchona* alkaloids have been performed. For instance, Mulder-Krieger et al. were able to show that some indole alkaloids, previously identified only in the leaves of *Cinchona ledgeriana*, also occurred in the stems and roots of living plants [15]. The presence of the indole alkaloids is explicable since they are precursors in the biosynthetic pathway of the quinoline alkaloids. The typical solvent systems described above, together with silica as a stationary phase, were utilised. Mass spectrometry was used as a confirmation of peak identity.

A final advantage of TLC is the comprehensive literature data, especially that published by Verpoorte and co-workers [18,52] which carefully details methods for separating the minor alkaloids as well as the major alkaloids, in conjunction with suitable spray reagents. In contrast, the HPLC literature detailing the analysis of the minor compounds is considerably more sparse. Thus, TLC in combination with specific spray reagents such as ferric chloride may still be the

method of choice, at least for qualitative analysis of the indole alkaloids [15].

The main disadvantages of TLC are the lack of automation, the semi-quantitative nature of most analyses, the lower separation efficiency in comparison with HPLC and the less reproducible nature of the stationary phase. No doubt many of these difficulties could be overcome at least partially by the application of high-performance TLC (HPTLC) methods, although this newer technique does not appear to have been specifically applied to the *Cinchona* alkaloids.

5. Capillary electrophoresis and capillary electrochromatography

Capillary electrophoresis (CE) is a technique useful for the separation of charged compounds, giving often higher efficiencies although often lower selectivity than HPLC. Capillary electrochromatography (CEC) is a hybrid technique which can be used for charged and uncharged compounds, combining the high efficiency of capillary electrophoresis with the high selectivity of HPLC. Analytes are transported through the column by the bulk flow of buffer caused by the electroosmotic flow (EOF), and separation may occur due to both electrophoretic and chromatographic mechanisms. A difficulty with the application of CEC to the analysis of bases such as the *Cinchona* alkaloids has been the rather low EOF generated by ''new generation'' RP materials when used in CEC [54]. Older generation silicas give good EOF but continue to give poor peak shapes for bases even in CEC. Recently, Lurie et al. [55] have demonstrated the analysis of quinine by CEC using an acidic phosphate buffer modified with acetonitrile on a traditional HPLC phase, in conjunction with hexylamine as a silanol masking agent. This technique emulates successful approaches with impure silicas in HPLC. Although some tailing of quinine was still reported, an efficiency of 10 000 plates was recorded on a 25 cm column, which is comparable but somewhat inferior to efficiencies obtainable by HPLC on the latest phases (Table 1).This rather low efficiency was attributable to significant tailing of quinine. Different selectivity effects were obtained for the same separations using capillary electrophoresis rather than CEC; tailing was still evident in these separations, which may be caused by detrimental interaction with silanol groups as in HPLC. To our knowledge, there have been no specific attempts at separation of the *Cinchona* alkaloids as a group by either CE or CEC; these methods have promise, but it remains to be seen whether difficulties associated with the analysis of basic compounds can be easily overcome.

6. Extraction and purification procedures

A number of extraction and purification methods have been reported, relating almost entirely to subsequent analysis of the extracts by HPLC, which as noted above, is the dominant final analytical method. Many different procedures have been applied to the extraction of samples connected with phytochemical studies. However, they have rarely been investigated in a rigorous fashion to determine their precision and accuracy. The commercial extraction of major alkaloids from *Cinchona* bark can be carried out by pulverising and grinding the bark, making it alkaline with lime and Soxhlet extracting in hot toluene. Other alkaloids may be recovered by further treatments [3]. The Bruxelles standard method for pretreatment and extraction of alkaloids from *Cinchona* bark for *analytical* purposes [56] was based on these commercial extraction procedures and was published in 1950 and widely adopted. This method involved grinding and sieving the bark, drying at 110 °C (at which temperature no loss of alkaloids was reported), followed by treatment with alkali and Soxhlet extraction with benzene. The method was established principally for the subsequent determination of quinine and cinchonidine using classical methods such as titrimetry and gravimetry. About 25 years later, Haznagy [57] claimed that there were problems involved in methods similar to the Bruxelles extraction method in that they gave variations in the amount of alkaloids extracted. It was suggested that the preliminary use of trichloroacetic acid in methanol was necessary, possibly to denature cellulose (although this effect was not proved unequivocally) and improve extraction efficiency. This was followed by treatment with base and benzene extraction as previously. However, this study was not performed

with the aid of HPLC as a final analytical procedure. Staba and Chung [58] adopted the Haznagy procedure for the extraction of alkaloids from cell suspension cultures although no evaluation of its efficacy was performed. A later study [26] compared the Bruxelles and Haznagy methods for *Cinchona* bark, but did not appear to show any significant increase in the quantity of alkaloids extracted using the latter procedure. Simple pretreatment of bark with calcium hydroxide followed by Soxhlet extraction with toluene or methanol was advocated [26]. Analysis of a bark sample subjected to this method of extraction is shown in Fig. 8. The relative standard deviation of the entire analytical procedure including extraction and HPLC analysis of the extract was reported as 4%. Recoveries of spiked bark samples were close to 100% for this method, although it was stated that such additions can never simulate the exact occurrence of alkaloids in the bark. Precision and accuracy of the analysis might be improved by the use of internal standards [33], however, finding compounds which are not naturally present in the samples, and yet simulate analyte behaviour without interfering in the chromatography of the extracts is difficult. Further study of all the issues is necessary, especially for ''newer'' sample matrices such as cell suspension cultures.

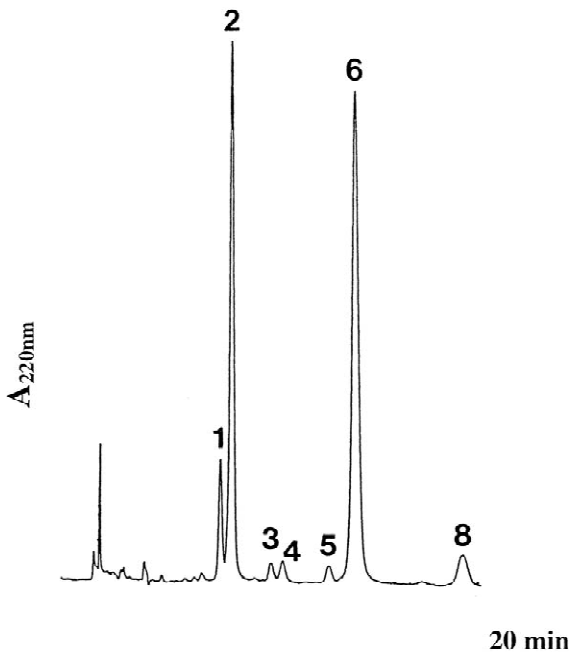

Fig. 8. HPLC chromatogram of *Cinchona calisaya* bark after extraction with toluene/alkali. For other conditions and peak identities, see Fig. 1 [26].

Purification methods for extracts have been applied, especially to cell suspension cultures containing low levels of alkaloids. In a typical procedure [59], cell samples were homogenised in 0.2 *M* sulfuric acid and washed with chloroform to remove impurities while the alkaloids are presumed to remain in the acid aqueous phase. The aqueous extract is made basic (e.g., with ammonium hydroxide) and again extracted with an organic solvent, this time to remove the alkaloids. Many variations on this theme have been proposed, although few if any procedures report the recovery of cell samples spiked with alkaloids. Thus, although these procedures seem eminently reasonable, and are likely to result in removal of those interferences which do not mimic the behaviour of the alkaloids, more work is necessary to assess their quantitative accuracy and reproducibility.

In biomedical studies, quinine and quinidine can be extracted from plasma or urine by making the sample basic with sodium or ammonium hydroxide and extracting into a solvents such as dichloromethane or diethyl ether [16,60]. Extracts can be concentrated by evaporation, and for the usual HPLC procedures, re-constituted in the mobile phase.

7. Conclusions

High-performance liquid chromatography in the reversed-phase mode using UV detection, is still the method of choice for the analysis of the *Cinchona* alkaloids. Good results may be obtained using ''new generation'' phases based on pure silicas, in conjunction with an acidic phosphate buffer (pH 3) modified with acetonitrile. Column efficiencies are variable due to the variety of complex solute–stationary phase interactions which can take place. However, many different packings have been evaluated for their performance, at least with quinine, allowing an informed choice of phase. Use of higher pH (e.g., pH 7) may give some useful selectivity effects, and raising the temperature above ambient has been shown to improve peak shape, especially at pH 7

[61]. Lowering the pH to 2.5 or even 2.0 may improve reproducibility of retention times when different batches of buffer are used (providing column stability is adequate). Silica bonded phases with ligands other than the usual C_{18} may give useful selectivity effects. Analysts should be careful not to overload columns, otherwise loss of efficiency and resolution for bases may result with as little as 0.5 μg of compound, if acidic mobile phases are used. Columns made with alternative RP materials such as polymeric materials or alumina phases give inferior results compared with silica. Normal phase chromatography on silica gives satisfactory results with alternative selectivity to RP separations. HPLC with mass spectrometric detection offers the best possibility of confirmation of peak identity or identification of minor alkaloids—however, relatively little specialist work has been reported in this area, especially with the more modern interfaces.

Gas chromatography has received very little attention for the separation of *Cinchona* alkaloids in recent years, due to the dominance of HPLC techniques. However, some re-investigation of this technique might be beneficial, to take advantage of the recent developments in column inertness and injection techniques. The greater availability and cheapness of GC–MS systems compared with HPLC–MS systems, and the greater structural information available from EI spectra generated from GC–MS is an incentive for further work in this area.

TLC is still a cheap alternative to HPLC, and can be useful for qualitative analysis, particularly for minor alkaloids, due to the wealth of information published on solvent systems and specific spray reagents for detection of these compounds. The use of high-performance TLC might further improve the attractiveness of this technique, although little if any work has been published on its specific application to the *Cinchona* alkaloids.

Capillary electrophoresis and capillary electrochromatography offer some potential for improvements in *Cinchona* alkaloid separations, although little specific application of these techniques has been made as yet.

Finally, although adequate sample preparation methods exist especially for biomedical analysis, where analysis of only a single administered alkaloid and human metabolites is required in body fluids, further work is necessary in this area for phytochemical work. Precise and accurate procedures need to be developed, especially for samples other than *Cinchona* bark, for which at least some detailed investigations have been made.

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