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Considerations of the behaviour of C18-chains and calixarenes and their application for determination of stationary phase volume in RP-chromatography

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Received April 14, 2008, accepted May 14, 2008

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Pharmazie 63: 619–627 (2008) *doi: 10.1691/ph.2008.8110*

The volume of stationary phase is extremely important for the estimation of thermodynamic information such as entropy and Gibbs free energy in HPLC. However, the definition of this volume strongly depends on the conditions chosen. In the present mini review the partitioning model of small molecules at low concentrations and methanol as modifier is assumed. All the other conditions that could influence the volume of the stationary phase like the thickness of the phase, the area of the phase, the order of C18-chains, the density of the chains, the temperature etc. had to be considered and are therefore reviewed in the present work. The results of the theoretical considerations and a new method of surface estimation were used to calculate the stationary phase volume.

1. Introduction

It is necessary to understand the interactions between analytes, stationary and mobile phase in order to develop optimal separation systems for RP-HPLC. A method commonly used to discuss these phenomena is the determination of the sorption enthalpy and the sorption entropy via van't Hoff plots. The equation commonly used to determine sorption enthalpy and sorption entropy is

$$\ln k = -\Delta H/(R \cdot T) + \Delta S/R + \ln \phi \qquad (1)$$

where k is the capacity factor, $-\Delta H$ is the sorption enthalpy, R is the gas constant, T is the absolute temperature, ΔS is the sorption entropy and ϕ is phase ratio (V_s/V_m). Therefore the phase-ratio of stationary and mobile phase (V_s/V_m) must be known to calculate the sorption entropy. The adsorption and the absorption are assumed as interaction mechanisms for our purpose: The characterisation of the stationary phase via enthalpy and entropy. Furthermore only methanol was used as the organic modifier.

The volume of the mobile phase has been examined (Melander et al. 1983; Smith et al. 1986; Engelhardt et al. 1984; Hennion and Rosset 1988). However it seems reasonable to calculate the volume of the stationary phase first and as a consequence deduce the volume of the mobile phase. Thus the volume of the stationary phase must be determined.

$$V_{\text{mobile phase}} = V_{\text{column}} - V_{\text{silicagel}} - V_{\text{stationary phase}} \qquad (2)$$

Previous methods of calculating the phase ratio have used the surface area of the bonded phase or have overestimated the volume by including that of the silica support. Guiochon and co-workers (Jandera et al. 1982) described the stationary phase as that part of the column volume which is not occupied by the mobile phase. Thus they included the silica support to the volume of the stationary phase. However, this at the first glance rough definition is useful for MIP systems because in that case the polymer skeleton is the stationary phase (Kim et al. 2006). Another description of the phase ratio was introduced by Kazakevich and co-workers (Kazakevich et al. 2001; Chan et al 2005). For a mobile phase including MeCN the volume of the pores is assumed as stationary phase volume. In that case MeCN precipitates at the surface of the bonded selectors and thereby no monomolecular layer but a multi-layer adsorption could be found. The layer has a thickness of approximately 14 Å and occupies 60% of the space available inside the adsorbent pores. Thus the volume of the pores and the volume between the silica particles can be understood as the stationary and mobile phase, respectively. But this behaviour of adsorption cannot be stated for methanol because merely a monomolecular adsorbed layer of approximately 2.5 Å could be determined. Thus only 12% of the space available inside the adsorbent pores is occupied. Hence the volume of the stationary phase also depends on the modifier which is used (Kazakevich et al. 2001; Chan et al. 2005). The objective of our work was the determination of the volume of the stationary phase using methanol as organic modifier. Therefore both theories are not suitable because for our purpose the volume of the stationary phase must vary with a change of the chain length of the selectors. And that would not be true using the first two theories.

Another definition has been given by Melander and Horvath (1980). Their approach defines the phase ratio as the ratio of the surface area and the void volume of the column. However, the type and the length of the selectors have also not been evaluated in this theory, implying that adsorption is the sole mechanism in RP-HPLC. This is, for normal modifier concentrations and small solutes, not the common opinion. For bigger solutes this ratio can be used because adsorption is assumed (Tessier 2002). But this condition is different from that of our tests.

Additionally, the last method includes the problem of the estimation of the surface area. The surface area can be calculated by the BET analysis method. However, the BET area is too big because C18-chains are not able to use the whole surface, which is captured by nitrogen. Only the surface area amenable for selectors is interesting for the estimation of the volume (V_s).

Sentell and Dorsey (1988) have defined the volume of the alkyl chains bonded to the silica surface as the volume of the stationary phase. This theory was postulated after validating the statistical, mechanical results of Dill (Ying et al. 1989), which showed that the principal retention mechanism for small nonpolar solutes is partitioning. Sentell and Dorsey (1988) accepted this idea of the partitioning model and they were able to show that retention time depends on bonding density. Thus the interaction of the solute in the stationary phase regardless of the selector (C18-chain or calixarenes) is the main interaction. If adsorption were the preferred interaction the bonding density and the kind of selector molecule should hardly influence the retention for small nonpolar solutes. Based on these results an equation was developed that uses the carbon content to calculate the surface coverage of C18-chains.

$$\alpha = \frac{\%C \cdot 10^6}{(12.011) \cdot (n_c) \cdot S) \cdot (100 - [\%C/(12.011) \cdot (n_c)] \cdot (M - 35.5)} \qquad (3)$$

Here α is the surface coverage ($\mu mol/m^2$), %C are grams carbon per 100 g bonded silica as obtained from elemental analysis, n_c is the number of carbon atoms per mole silane, M is the molecular weight of the silane and S is the surface area of the native silica gel in m^2/g.

The term $(100 - [\%C/(12.011) \cdot (n_c)] \cdot (M - 35.5))$ is a correction factor that helps to estimate the native silica surface. $C\%/(12.011 \cdot n_c)$ is comparable with a molecular weight of the selector that only considers the carbon skeleton.

The volume can be calculated by Eq. (4)

$$V_s = \alpha \cdot S \cdot (100 - [\%C/(12.011) \cdot (n_c)] \times (M - 35,5)) \cdot v \cdot W_p \cdot 10^{-6} \qquad (4)$$

where $100 - [\%C/(12,011) \cdot (n_c)] \cdot (M - 35,5)$ is the correction factor for the native silica gel again, W_p is the mass of the column packing and v is the molar volume of the bonded alkyl group in cm^3/mol. The last parameter mentioned is not easy to investigate. Sentell and Dorsey (1988) have used the results of Cheng (1985). He analyzed the density of chromatographic selectors using gravimetry. With the density of the chromatographic selector and its molecular weight it is simple to calculate the molar volume.

The volume of the alkyl chains has been acquired by investigating the densities of native silica gel and alkyl bonded silica gel. For that purpose methanol was added to the dry silica gels (native and alkyl bonded) to an exact mark and the weight difference of native and alkyl bonded silica gel was used for the calculation.

Based on the theory of Sentell and Dorsey a new definition of stationary phase is presented where not only the selectors but also the adsorbed liquid molecules and less covered areas define the volume.

Sentell and Dorsey (1988) determined the molar volume of the C18-chains to calculate the volume of the stationary phase. However the space occupied by the stationary phase, does not only consist of the molar volume of the C18-chains but also of ad- and absorbed molecules of the mobile phase and of space not occupied by the chains (definition of the phase volume) (Sander and Wiese 1993).

Molecules of the mobile phase are absorbed into the matrix and let the volume swell (Ban et al. 2004; Klatte and Beck 1996). Interestingly the composition of the adsorbed molecules does not match those of the mobile phase. The percentage of methanol of the methanol/water mixture, adsorbed in the matrix, enhances with an increasing penetration into the C18-Matrix to 100% (Klatte and Beck 1996).

The unoccupied space in the C18-phase arises because of the different C18-densities on the silica gel. It depends on the manufacturing method which is used because, depending on the method, areas with high C18-density alternate with areas of lower density (Liu et al. 2001). Additionally using the calculation of molar volume and including the coverage density unoccupied spaces occur, even with equal distribution.

In order to define the term "volume of stationary phase" the most important items will be defined. The results of the theoretical examination and own experimental results will be presented and a new method of calculation for the stationary phase volume will be shown.

2. Distribution of C18-selectors on silica gel

One possible C18-distribution was presented by Lochmüller et al. (Lochmüller and Wilder 1979; Lochmüller et al. 1983). They defined a part of the stationary phase as groups of selector associates which behave similarly to liquids. Gilpin et al. (Gilpin and Squires 1981; Gilpin et al. 1982) postulated two possible states (bristle and folded) and related it to the state of the temperature and the solvent. Liu et al. (2001) determined a phase structure dependent on the water content during the syntheses. The higher the water content during syntheses had been the more "islands" of C18-selectors were built. The unoccupied space was covered equally in the next steps of the syntheses. Thus areas existed with high and low C18-densities. These results were supported by a work of Gritti and Guoichon (2004). They investigated the accuracy of adsorption isotherm parameter using dynamic HPLC methods and found that the stationary phase is not homogeneous.

3. Molar volume of the C18-selectors

The molar volume of the selector states the volume which is occupied by one mole of it. For amorphous, not crystalline or liquid crystalline states assumed in HPLC, it is difficult to determine a volume. In order to quantify the molar volume there are experimental and theoretical approaches. An experimental method was introduced by Cheng (1985). A theoretical approach is to define the C18-molecule as a cylinder and to assume that one mole consist of 6.022×10^{23} cylinders.

The height and the diameter must be known to calculate the cylinder. The molecule diameter calculated using the theoretical length of the C–C-bonding is ~100 pm and the resulting molecule length is 2340.7 pm.

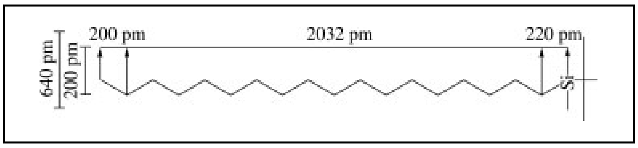

Fig. 1: Height of a C18-chain

However it is known that the last methylene group of alkyl chains captures a space with 200 pm diameter. Additionally it is known that the length of an all-trans alkyl chain is expanded about 127 pm by a $CH_2{-}CH_2$-group. A new calculation with these results yields a maximal length of 2452 pm for an all-trans dimethyloctadecylsilyl molecule. The diameter of the hypothetical cylinder must be enhanced to 200 pm because van der Waals interactions with analytes, which are the most common interactions in HPLC, already occur in that distance. Thus an ideal cylinder with a diameter of 200 pm and a length of 2452 pm was determined (Fig. 1). The last significant factor for the volume is the silyl group. It has two methylene groups and therefore its diameter must be enhanced by about 440 pm (radius methylene group $2 \times (100\ \text{pm})$ + length of silica carbon bonding $2 \times (220)$ pm s.a.). With all these results a total volume of the C18-selector of 141 Å^3 can be determined. The area occupied by a selector molecule is 32 Å^2 due to the dimethylsilyl structure and the assumption of free rotatability. A volume of 599 Å^3 can be calculated using the experimental results of Cheng (1985). Assuming the same length of the selector molecule an area of 25 Å^2 can be calculated and this results in a diameter of 5.6 Å. Kazakevich (2007) presented a molecular volume of 630 Å^3 for a C18-ligand (Table 1). Using our maximal selector length an area of 25.7 Å^2 is occupied by one molecule (calculations of Kazakevich were done using a length of 24 Å with a result of 26.2 Å^2) and the area has a diameter of 5.7 Å. These results show that the diameter of the selector can be quoted with 6 ± 4 Å. But they also show that the amount of the molar volume is not exact and depends on the method used. The big difference between our volume and the others is caused by the calculation. We divided the C18-chain in two cylinders. The first was the $Si(CH_3)_2$ group with a diameter of 640 pm and a height 220 pm. The second was the $(CH_2)_{17}{-}CH_3$ group with a diameter of 200 pm and a height of 2032 pm. All the other calculations were done with the diameter of the first group and the length of the whole molecule.

The average distance between the C18-chains was determined, in order to get a picture of the coverage of the stationary phase. Remarkably the average distance between two chains, assuming a density of 1 μmol/m^2, was 1454 pm. With this result it can be shown that, assuming a diameter of interaction of 200 pm for each chain, free space between them must occur where no interactions with the chains take place. The supposed regular distribution of the selectors is not always correct in practice. Liu et al. (2001) could show that the stationary phase is heterogeneous with areas of low and high densities. Furthermore chain density is limited by the steric hindrance of the dimethylsilyl groups. Thus there is a maximum coverage of about 4 to 6.6 μmol/m^2 in dependence on the surface area which is assumed. The theoretical densities can be calculated by the area which is occupied by one selector. However, a penetration of this theoretical area is possible and would lead to a higher bonding density (Fig. 2).

Table 1: Calculation of volumes and areas of a C18-selector

Volumes of one selector in Å^3	Diameter of the covered area on the silica matrix of a C18-molecule in Å	Area of a selector in Å^2
141	6.4 (Meyer)	32.2
630	5.7 (Kazakevich)	25.7
599	5.6 (Sentell, Dorsey)	24.5

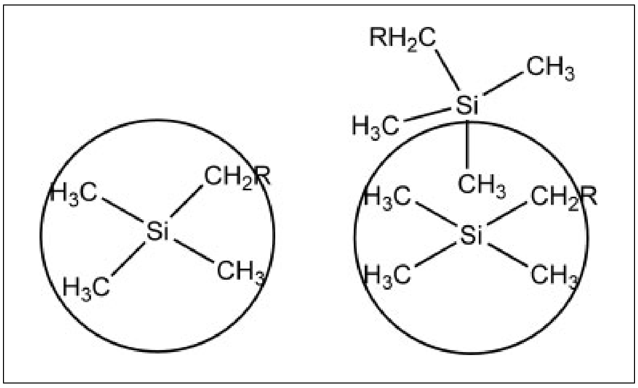

Fig. 2: Possible overlaps of the areas of two selectors

With these facts of less average C18-chain-density the less steric selectivity of monomeric C18-phases can be explained because it is not significant whether the analyte is rigid or flexible or spherical in order to penetrate into the stationary phase.

The areas of high density as well as those of low density are a part of the stationary phase. Therefore not only the molar volume of the selectors but also the free space between the chains defines the stationary phase. Additionally liquid molecules are adsorbed at or into the stationary phase (Ban et al. 2004; Klatte and Beck 1996).

Thus the stationary phase can be defined as a compact phase and its volume can be calculated by its thickness and the area occupied by it.

4. Area of stationary phase

The area occupied by the stationary phase is defined by the surface of the media (silica gel). Thus the surface area, accessible for the selector, is the parameter which is used to calculate the volume.

The standard method to measure the surface area of HPLC-silica gel is the BET-method. This method uses nitrogen to estimate the surface because nitrogen is small and therefore it can enter almost every volume. Additionally it forms a monolayer and with the knowledge of the area occupied by an atom of nitrogen (16.2 Å^2) the calculation of the surface area is possible. The BET-surface however is not that exact for the volume calculation mentioned above, because nearly the whole surface of the silica gel is determined and not only that part which can be occupied by the stationary phase.

The surface area had to be estimated by a method that considers this fact. Additionally it must be mentioned that the surface of the silica is rough and the macro- and micro-pores (<50 Å and 10 Å respectively) are probably cylindrical. Therefore the picture of a plain area where the chains are bonded at is incorrect. This curvature of the pores causes a reduction of the volume even with an all-trans conformation.

The surface area which is estimated through CTAB (cetyltrimethylammonium bromide) gives attention to all these demands because the size of the molecule is similar to that of a C18-molecule. Therefore the micro pores which are not coverable by C18-selectors will not be detected by the CTAB molecule and their surface will not be measured. Additionally a defined area for each CTAB molecule is used in the calculation of the surface area (similar to BET). That means the surface of a covered plane assuming the highest possible density can be calculated by multiplying the area of a CTAB molecule by their number. The highest possible density in a pore is less than that of a plane due to the curvature because the molecules hinder each other. The calculation of the surface, however, implies a plane because the area of a CTAB molecule on a plane is used. Thus the surface of a pore is estimated smaller than it actually is. This area not included in the measurement is exactly that area which cannot be covered by the C18-selectors.

The CTAB method (ASTM D: 6845-02) is protected and thus cannot be described here (ASTM, 2002).

The CTAB-surface gives attention to all demands of a C18- and calixarene-covered area because the steric hindrance and the pore curvatures are considered.

5. Thickness of the stationary phase

Knowing the surface area of the stationary phase the volume depends on the thickness of the compact phase. The thickness is defined by some parameters (ability to swell, organization of the selectors and with this the conformation and orientation of the selectors).

Different working groups have developed different models about the shape of C18-chains and different theories about the transformation of the phases influenced by varying parameters.

The most important parameters were reviewed, discussed and complemented with own investigations.

5.1. Methanol and the stationary phase

The behaviour of methanol, of methanol/water mixture, and C18-chains were investigated by Montgomery et al. (1992). It could be shown that the structure of the chains is not influenced by the alcohol but that only the surface is wetted. Bliesner and Sentell (1993) proved that Methanol precipitates on the selectors of the stationary phase using NMR. Klatte and Beck (1996) could show the concentration enhancement of methanol at the stationary phase depending on the depth of penetration with a computer model. Additionally they confirmed that the structure of the stationary phase will not change using a methanol range of 10–90% methanol as organic modifier in the mobile phase (Klatte and Beck 1996). Furthermore it is interesting that the methanol molecules which are precipitated on the stationary phase are highly ordered. Sun et al. (2006) ascertained that the methyl groups of the absorbed methanol molecules orientate towards the lipophilic chains, whereas the polar hydroxyl group points towards the solvent side. Klatte and Beck (1996) confirmed the theory of ordered methanol und postulated that the methanol molecules start to orientate in a distance of 20 Å from the silica surface (~3 Å above the C18-chains). This result is in excellent agreement with those published by Kazakevich and co-workers (Kazakevich et al. 2001; Chan et al. 2005). Graphically Ban et al. (2004) showed the behaviour of solvent molecules of the mobile phase and the behaviour of the stationary phase by varying methanol concentrations. A complete separation of stationary and mobile phase could be shown at a methanol range of 0 to 10%. It was outstanding that the stationary phase did not collapse. After enhancing the methanol concentration over 10% a penetration of mobile phase into the stationary phase could be shown. However, the inner space of the stationary phase was not wetted by the solvent. The incomplete wetting of the phase is consistent with former results (Ban et al. 2004). Sander et al. (1983) used IR-methods to relate the degree of disorder of the chains to the methanol concentration of the stationary phase. These results showed that the order of methanol wetted phases is higher compared to results measured in vacuum, but the order does not change in a range between 70 and 100% methanol.

Methanol wets the hydrophobic C18-chains and orientates the methyl group towards the alkyl groups and the hydroxyl group towards the solvent in stationary phase environment. However, methanol does not destroy the structure of the chains in normal modifier concentration and only penetrates the stationary phase up to a depth of 10 Å (Klatte and Beck 1996). The adsorbed liquid molecules enhance the volume of the stationary phase up to 3 Å because adsorbed, ordered molecules do not belong to the mobile phase anymore. These results are not valid for all modifiers but only for methanol because MeCN for example has other wetting properties and will react differently.

5.2. Order of the stationary phase

The order of the C18-chains is, beside the precipitation of methanol, the most essential factor for the structure and the thickness of the stationary phase. All other parameters influence the order only indirectly by the amount of disorder they cause. Assuming a maximal ordered phase all selectors would exist in the all-trans conformation with a resulting phase thickness of 24.5 Å + 3 Å. When the order and with it the structure changes the thickness of the selectors will be reduced.

For considering the order of the stationary phase, first the selectors will be described in the condition of maximal order and then in condition of disorder.

A C18-phase has a maximal order when all chains are in the all-trans conformation and the chains are arranged parallel with the same distance similar to a crystal structure. The amount of disorder increases when the all-trans conformation changes into a gauche-form. This occurs with chains longer then 10 carbon atoms. However a distinction between some conformation changes is necessary. A single gauche-conformation changes the order differently, in dependency on the carbon atom, where the change of conformation takes place. Therefore it follows that chains with a conformation change at the proximal ending increase the disorder of the phase intensively whereas gauche conformations at the distal ending (end gauche) influence the disorder less because the morphology of the molecule is not modified strongly. If two gauche-conformations per molecule occur and they have the opposite direction then a kink conformation is exhibited. When both conformational changes point into the same direction the selector will adopt an U-shape conformation and increase the disorder most (Fig. 3).

The frequency of the occurrence of the different conformations (all-trans, gauche, end gauche, kink) has to be estimated in order to determine the order of a C18-phase. This was accomplished in dependence on temperature

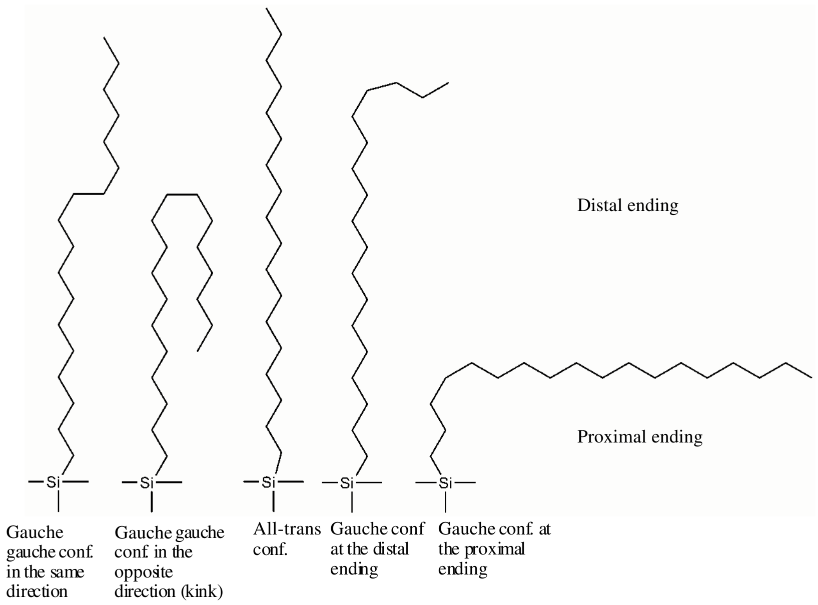

Fig. 3:
Possible types of conformation of a C18-phase

(Ban et al. 2004; Sander et al. 1983; Srinivasan et al. 2006), chain length (Sander et al. 1983; Srinivasan et al. 2006) and coverage density (Lippa et al. 2005).

5.2.1. *C18 chains and its behaviour in high aqueous environment*

Many theories have been put forward and works have been done which have tried to explain the structure, shape and behaviour of the C18-chains. There are common and special considerations which deal with C18-molecules in polar environment.

Halasz and co-workers (Karch et al. 1976) published the "bristle structure" where the chains are orientated vertically like bristles towards the silica surface. Hemetsberger et al. (1979) supposed another structure with collapsed chains aspiring to a minimal surface. A similar picture was drawn by Harris and co-workers (Wong et al. 1991). They found collapsed phases via fluorescence measurement at high water content. Mountain and Thirumalai (2003) also showed folded hydrocarbons. They determined n-eicosane via molecular dynamics simulations and found chains with end to end contact to be about 12 times more likely than unfolded conformations.

However, Tolls et al. (2002) published that long-chain hydrocarbons prefer the all-trans conformation in highly polar environment. This conclusion was supported by Cramer and Truhlar (1992). Using a Monte Carlo simulation Sun et al. (2006) presented results demonstrating that most of the C18-molecules are not folded on the surface but are extended into the space with an average length of 14.8 ± 4 Å. However, the lengths of the selectors referring to the cumulative number do not show an ideal Gaussian distribution. A little maximum at 5 Å end to end distances exists. At this distance the chains show an U-shape conformation and with it the maximal folded conformation. This peak at 5 Å exists also at mobile phase mixtures of 33/67% and 66/24% methanol/water but not in such a distinctive way. The second peak at 17 Å (16 Å < > 18 Å) also occurs at each mobile phase composition. This means that a change in the mobile phase does not affect the thickness of the stationary phase. Only the compositions of the conformations of the selectors will change. This outstanding result was supposed by Ban et al. (2004) using molecular dynamic computer simulation. Again the thickness was determined with 17 ± 3Å independent of the mobile phase composition.

Recapitulatory it can be pointed out that water content over 90% in the mobile phase leads to strong modification of the stationary phase. However it must be emphasized that high water content does not lead to a completely folded or collapsed stationary phase. The structure of the alkyl-chains will change. However, the thickness and with it the volume will hardly alter.

5.2.2. *Influence of temperature on the stationary phase*

Sander et al. (Sander et al. 1983; Sander et al. 2001) investigated the temperature as an influencing parameter on the alkyl chains and could show positive results. Srinivasan et al. (2006) also found a modification of the number of gauche and kink conformations per chain over a temperature range of 180–340 K. A slight increase of order could be shown with falling temperature. This tendency was confirmed by Ban et al. (2004). The results were, however, more exact. Furthermore they differentiate the investigations even more by classifying the C18-chain into three parts (root, middle, end) and by determining the order of these three parts separately. All these results show that temperature influences the order of the chains. As mentioned, in the amount of order is controlled by the conformations. Therefore it is evident that rising temperature causes a slightly higher amount of modified conformations. It is noticeable that the average location of the modified conformation is dependent on the temperature. The disorder enhances with rising temperature mainly on the basis of the chains. This fact is significant for the carbon density inside the phase because modifications of the chains on the basis cause strong morphologic changes so that carbon atoms concentrate at the proximal region of the phase.

These results were supported partly by Singh et al. (2002). They deuterated the C18-chains on position C_4, C_6, C_{12} and measured the gauche-defects in dependency on the temperature. A higher amount of modified chains on the proximal ending was unexpectedly found with increasing temperature. This seems to be an antagonism to results of Ban et al. because their results showed that the order used to be higher at the proximal part than at the middle or end parts but changed mainly with rising temperature on the proximal part. However, if the conditions and results of Ban et al. (2004) are considered at 100% water, the highest amount of gauche defects can also be measured at the proximal ending. Furthermore the allocation of the solute in root, middle and end does not agree with the allocation of the selector by means of the three deuterated carbons. Basically, Singh et al. (2002) also determined the positive influence of decreasing temperature on the order of the phase.

The influence of temperature on the order is proven. If the temperature decreases, the amount of disorder also decreases but slowly and continuously (Sander et al. 2005). Therefore it must be assumed that no escalate phase transition exists. The increasing order of the phase is used in HPLC to separate steric isomers because the slight energetic difference in the retention of these isomers can be used in systems with a degree of high order. This is caused by the energy which is needed to form a cavity in the C18-chains. More rigid molecules like triphenylene and more flexible molecules like o-terphenyl require different cavities although they are almost identical. The energy difference is more distinctive with ordered phase. Thus separation is possible or the selectivity is better (Sander et al. 2005).

5.2.3. Influence of the density on the stationary phase

Beside the temperature and the selector length the order of stationary phase is affected by the coverage density of C18-chains (Klatte and Beck 1996; Lippa et al. 2005; Sander et al. 2005).

Lippa et al. (2005) could show that the number of gauche conformations decreases with increasing coverage density using molecular, dynamic simulations of covered silica gel with a density of 1.64 to 5.94 μmol/m^2. Sander et al. (2005) also found supporting results for that theory and confirmed them using Fourier transform infrared spectroscopy, Raman spectroscopy and NMR. Lippa et al. (2005) differentiated their results even more by relating the number of gauche-conformations to the position of carbon atoms. It is evident that most of the gauche defects appear in the range between the fourth and the eighth carbon atom independently of the coverage density. This fact may be responsible for the different results discussed above.

A shift of carbon density of the stationary phase to the distal ending is affected by the increasing order caused by the enhanced coverage density. Furthermore it could be shown that the distribution of the carbon concentration along the phase axis equals due to increased densities and that very high densities cause a thicker stationary phase (Lippa et al. 2005). However, very high densities are not possible in practice of monomeric phases. The calculated thickness for phases used in practice was 16 ± 3 Å and supports the results of Sun et al. (2006) and Ban et al. (2004).

The coverage density influences the order and with it the distribution of carbon atoms along the phase axis. A higher density causes a higher degree of order and a shift of the carbon concentration towards the distal ending.

6. Recapitulatory notes on C18-chains

The thickness of stationary phase is influenced by different parameters (water content of mobile phase, properties of organic modifier, temperature and coverage density of stationary phase).

A water content of the mobile phase higher than 90% leads to a modification of the structure of the stationary phase. The thickness however does not change (Sun et al. 2006). At this point it must be considered that the phase interface, as it is needed for the volume definition, does not comply with the average length of the selectors. In fact the length, ascribed to most of the chains, must be considered. This information can be obtained by plotting the number of alkyl chains against the length of the chains. The diagram shows an asymmetric dispersion with a maximum at 17 ± 3 Å (Ban et al. 2004; Sun et al. 2006).

Methanol does not lead to a structural change of the stationary phase. On the other hand the degree of order increases using methanol compared to results measured in vacuum (Sander et al. 1983). But this is only true for that case because a further enhancement of methanol concentration does not cause a significant influence on the order of the chains. The second and outstanding property of methanol, concerning the phase volume, was the precipitation on the stationary phase (Bliesner and Sentell 1993). This phenomenon could be found until a distance of 3 Å above the C18-chains (Kazakevich et al. 2001; Chan et al. 2005; Klatte and Beck 1996). Additionally these methanol molecules orientate their CH_3-groups towards the lipophilic chains and their hydroxyl groups towards the polar solvent. The adsorbed molecules do not longer belong to the mobile phase and thus have to be added to the stationary phase. Thus the measured phase thickness has to be enhanced about 3 Å.

The temperature influences the order of the C18-solutes. If the temperature decreases the degree of order increases (Sander et al. 2005). The effect on the thickness however is marginal because only a shift of carbon concentration to the distal ending can be observed. Thus the properties of the stationary phase change but not its volume. It is notable that the effect of temperature is small and continuous. Therefore a large temperature range has to be considered to ascertain a measurable change of order.

The coverage density has a similar importance as the temperature for the degree of order of the phase. Increased densities enhance the degree of order because the interactions of chains with gauche defects become energetic more inefficient (Lippa et al. 2005). A lot of work has been done on the basis of this theory using both simulations (Klatte and Beck 1996 1995; Wick et al. 2004) and measurements of covered silica gels (Liu et al. 2001). A comparison of results of both approaches is difficult because the values of covered silica gel are determined using the BET surface and thus are not that exact. Furthermore areas of high and low coverage density could be found (Liu et al. 2001). Thus the density is just an average value. The thickness of about 17 ± 3 Å is not influenced by the densities used in monomeric stationary phases similar to the temperature. Solely the shift on the carbon density to the distal ending could again be observed at enhanced order (Lippa et al. 2005).

The thickness of the stationary phase used for the calculation of the volume is assumed with 17 ± 3 Å $+ 3$ Å $=$ 20 ± 3 Å.

7. Calixarenes

Calixarenes, following cyclodextrines and crown ethers, are the third generation of supramolecules used in HPLC

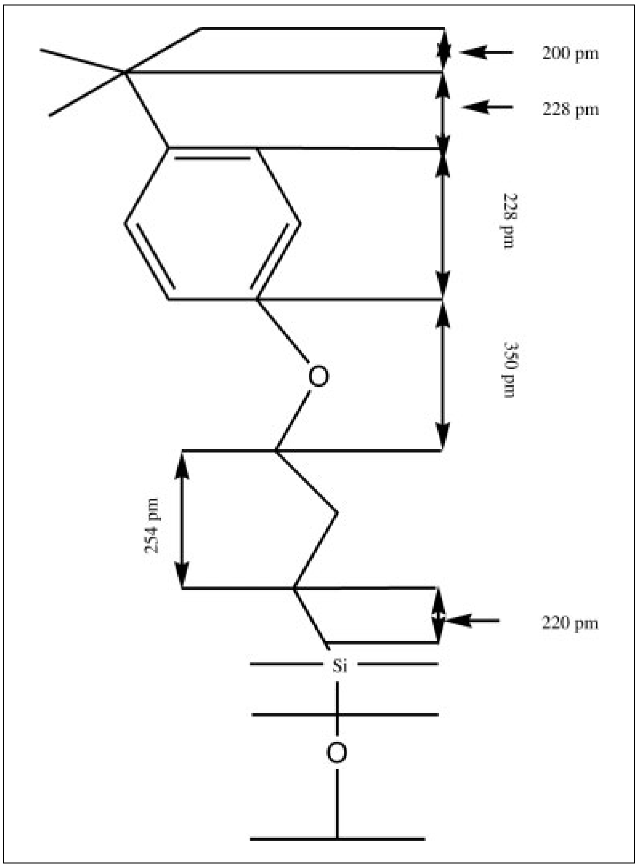

Fig. 4: Thickness of a calixarene

Table 2: Examples of surface ratios

BET-surface (m^2/g)	150	146	157	145	157	134	133
CTAB-surface (m^2/g)	116	120	124	118	123	118	116
Percent ratio (%)	77.3	82.2	78.9	81.4	78.3	88.1	87.2

as stationary phases (Fig. 4). They consist of phenol units linked via methylene bridges, can form inclusion complexes and are used in many fields of science (Meyer and Jira 2007). The reflections which were made for the C18-chains partly match the calixarene phases and they should be verified for their validity. The CTAB surface is amenable as well for calixarenes as for C18-molecules. Therefore these data can be used.

The other parameters (mobile phase, temperature, coverage density) probably have less bearing on the calixarenes than on the C18-chains because these molecules have a high inner order which is hard to affect. The water content of the mobile phase could influence the calixarene-bonded phases because the calixarene molecules also exist in different conformations (cone, partial cone, 1,3 alternate…). However, these modifications do not affect the thickness of the stationary calixarene-bonded phase because at each conformation, phenol units of the calixarenes are orientated axially towards the spacer and thus show the same phase-interface as cone calixarenes. Additionally the calixarene spacers are that short that an all-trans conformation must be assumed.

The thickness of the adsorbed layer of methanol is probably the same for calixarenes and alkyl chains because results of investigations on phenyl phases also showed a thickness of the adsorbed layer of 2,5 Å.

The height of calixarenes was calculated with 11 + 3 Å and 15 + 3 Å for the calix[n]arenes and p-tert.-butylcalix[n]arenes, respectively (Fig. 5).

8. Volume of the stationary phase

The CTAB-surface of the silica gel used is 224 m^2/g (experimental see ASTM 2002). As expected, this surface is smaller than those which were determined by the BET method (320 m^2/g). The percent ratio of BET and CTAB surface is 72% and matches the examples given by the owner of the method (Table 2).

The CTAB surface area is valid for bare silica gel. Therefore absolute surfaces have to be acquired for each column depending on the carbon load. Thus phases with equal selectors but different carbon load must have different phase ratios. The mass of a column filling is given with 1.05 g. The mass of bare silica gel can be calculated with Eq. (5).

$$m_f = \{100 - [\%C_s/(12.011 \cdot n_{cs}) \cdot M_s] - [\%C_{Si}/(12.011 \cdot n_{csi}) \cdot M_{Si}]\} \cdot 10^{-2} \cdot m_p \quad (5)$$

where $\%C_s$ is the carbon load of the covered silica gel caused by the selector, $\%C_{si}$ is the carbon load of the covered silica gel caused by endcapping, n_{cs} is the number of carbon atoms of the selector, n_{csi} is the number of carbon atoms of the endcapping material, M_s is the molar mass of the selector, M_{si} is the molar mass of endcapping material and m_p is the mass of the column filling.

Thereby the mass of the selectors and endcapping materials is subtracted from the mass of the column filling. The individual surface of column filling and with it the stationary phase volume can be calculated in Table 3.

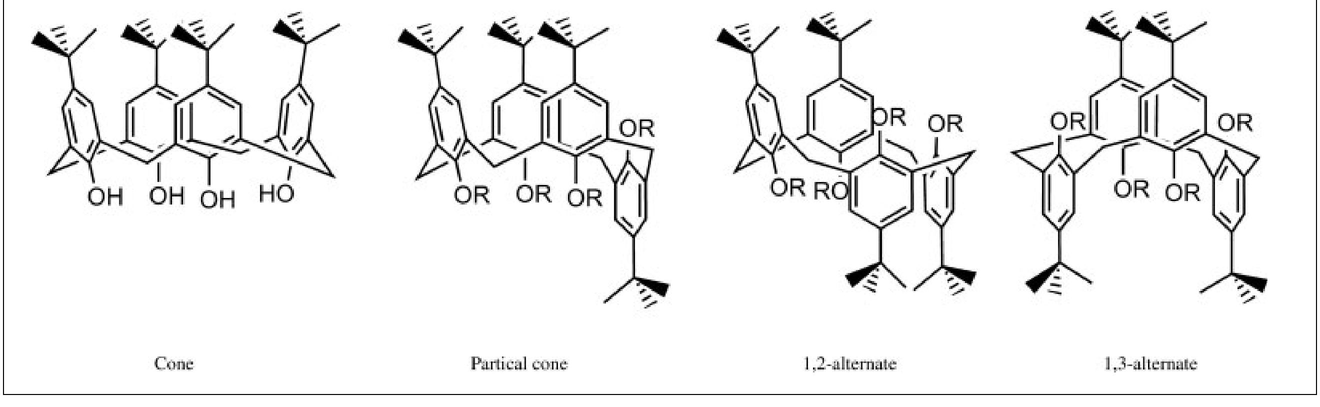

Fig. 5: Conformations of calix[4]arenes

Table 3: Values for the calculation of the stationary phase volume

	Kromasil	AI	AII	AIII	BI	BII	BIII
Molar mass (g/mol)	311.3	582.5	875.16	1168	810.5	1211.1	1624
Carbon load (%C for the selector)	17	11.3	13.2	11.7	12.9	14.2	15
Mass of silica gel (g)	1.05	1.05	1.05	1.05	1.05	1.05	1.05
Mass of pure silica gel (g)	0.753	0.824	0.800	0.819	0.805	0.789	0.778
Number of C-atoms	20	40	60	80	56	84	112
Surface area (m^2)	168	184	179	183	180	176	174
Volume of silica gel (mm^3)	428	468	454	465	457	448	442
Volume of stationary phase (mm^3)	337	258	250	256	330	323	319

The values of the columns investigated by us are shown (table 3). Continuative calculations of the volume of mobile phase show that the calculated volumes match those obtained by the void volume.

9. Conclusion

The definition of stationary phase depends on the conditions used in HPLC. Our purpose was the definition and estimation of the volume of stationary phase for small compounds at low concentrations using methanol as organic modifier. Under these conditions the stationary phase must be considered as a compact phase including not only the selectors but also the liquid molecules and the space in it. Therefore its volume can be calculated with the covered area and the thickness. The thickness and the order of the phase are affected by some factors which were reviewed:

- The methanol content of mobile phase only influences the structure. Thus the order is less influenced. The thickness, however, is increased by the methanol through precipitating on the stationary phase.
- The order of the phase represents a very important or maybe the most important effect on the thickness of the phase because, except methanol, all the other parameters affect the order. It is evident that the carbon density adjusts when the order is increased. And the thickness is enhanced by very high coverage density.
- The temperature influences the order in such a way that decreasing temperatures determine a degree of higher order. However the temperature range must be chosen widely to observe a modification because the influence is small but continuous.
- The coverage density affects the order in such a way that an increasing density causes a degree of higher order because gauche conformations become energetic more unfavourable.
- The concentrations of methanol in the mobile phase below 10% are inadequate to wet the stationary phase. Hence the polar character of the water affects the structure of the chains and a total separation of the two phases (mobile and stationary) can be observed.

  The CTAB method for calculation of the volume of stationary phase is useful. The CTAB surface represents the area which is covered by the stationary phase more exactly than areas which were acquired by the BET method.

  With these results thermodynamic determinations in HPLC can be calculated more accurately to improve the understanding of interactions in HPLC.

10. Experimental

10.1. Columns

The six calixarene-bonded columns (Caltrex® AI-III and Caltrex® BI-III) and the Kromasil® C18-column (Eka Nobel) were obtained from Syntrex GbR (Greifswald, Germany) with a carbon content of 14.3% C; 16.2% C; 14.7% C; 15.9% C; 17.2% C; 18% C; 20% C, respectively. The dimension of the columns was 125 × 4 mm I.D. All columns were packed with Kromasil® silica gel (5 μm).

10.2. CTAB method

The technique we used to determine the CTAB surface is a standard method of ASTM with the number ASTM D: 6845-02 (ASTM 2002; Sandrine et al. 2005). This standard is used to determine the rough surface area of silica in rubber industries. The standard is protected. Therefore we are not allowed to publish the details of the method.

References

ASTM D 6845-02 (2002) Standard test method for the determination of the surface area of precipitated silica using CTAB, ASTM New Standard Vol. 09.01.

Ban K, Saito Y, Jinno K (2004) Characterization of the microscopic surface structure of the octadecylsilica stationary phase using a molecular-dynamics simulation. Anal Sci 20: 1403–1408.

Bliesner DM, Sentell KB (1993) Deuterium nuclear magnetic resonance spectroscopy as a probe for reversed-phase liquid chromatographic bonded phase solvation. 2. Aqueous solvation in methanol and acetonitrile binary mobile phases. Anal Chem 65: 1819–1825.

Chan F, Yeung LS, LoBrutto R, Kazakevich YV (2005) Interpretation of the excess adsorption isotherms of organic eluent components on the surface of reversed-phase phenyl modified adsorbents. J Chromatogr 1082: 158–165.

Cheng W (1985) Differential density method for determination of carbon load on chromatographic packings. Anal Chem 57: 2409–2412.

Cramer CJ, Truhlar DG (1992) An SCF solvation model for the hydrophobic effect and absolute free energies of aqueous solvation. Science 256: 213–217.

Engelhardt H, Müller H, Dreyer B (1984) Is there a “true” dead volume for HPLC columns? Chromatographia 19: 240–245.

Gilpin RK, Gangoda ME, Krishen AE (1982) Effect of conditioning solvent on the orientation of bonded hydrocarbon moieties in totally aqueous mobile phases. J Chromatogr Sci 20: 345–348.

Gritti F, Guiochon G (2004) Accuracy and precision of adsorption isotherm parameters measured by dynamic HPLC methods. J Chromatogr A 1043: 159–170.

Gilpin RK, Squires J A (1981) Effect of temperature on the orientation of bonded hydrocarbon phases in totally liquid chromatography systems. J Chromatogr Sci 19: 195–199.

Hemetsberger H, Behrensmeyer P, Henning J, Ricken H (1979) Reversed phase, high-performance liquid chromatography: Effect of the structure of the chemically bonded hydrocarbon ligand on retention and selectivity. Chromatographia 12: 71–76.

Hennion MC, Rosset R (1988) Void volume determination and experimental probes in reversed phase chromatography. Chromatographia 25: 43–50.

Jandera P, Colin H, Guiochon G (1982) Interaction indexes for prediction of retention in reversed-phase liquid chromatography. Anal Chem 54: 435–441.

Karch K, Sebastian I, Halasz I (1976) Preparation and properties of reversed phases. J Chromatogr 122: 3–16.

Kazakevich Y (2007) In: HPLC for Pharmaceutical Scientists; Kazakevich Y, LoBrutto, Rosario, Ed.; John Wiley & Sons Inc.: New Jersey, p 106.

Kazakevich YV, LoBrutto R, Chan F, Patel T (2001) Interpretation of the excess adsorption isotherms of organic eluent components on the surface of reversed-phase adsorbents: Effect on the analyte retention. J Chromatogr 913: 75–87.

Kim H, Kaczmarski K, Guiochon G (2006) Isotherm parameters and intraparticle mass transfer kinetics on molecularly imprinted polymers in acetonitrile/buffer mobile phases. Chem Engineering Sci 61: 5249–5267.

Klatte SJ, Beck TL (1993) Temperature-dependent behavior in a high-density chromatographic system. J Phys Chem 97: 5727–5734.

Klatte SJ, Beck TL (1995) Molecular dynamics simulations of tethered alkane chromatographic stationary phases. J Phys Chem 99: 16024–16032.

Klatte SJ, Beck TL (1996) Microscopic simulation of solute transfer in reversed phase liquid chromatography. J Phys Chem 100: 5931–5934.

Lippa KA, Sander LC, Mountain RD (2005) Molecular dynamics Simulations of alkylsilane stationary-phase order and disorder. 1. Effects of surface coverage and bonding chemistry. Anal Chem 77: 7852–7861.

Liu Y, Wolf LK,. Messmer MC (2001) A study of alkyl chain conformational changes in self-assembled n-octadecyltrichlorosilane monolayers on fused silica surfaces. Langmuir 17: 4329–4335.

Lochmüller CH, Colburn AS, Hunnicutt ML, Harris JM (1983) Organization and distribution of molecules chemically bound to silica. Anal Chem 55: 1344–1348.

Lochmüller CH, Wilder DR (1979) The sorption behavior of alkyl bonded phases in reversed-phase, high performance liquid chromatography. J Chromatogr Sci. 17: 574–579.

Melander, WR, Erard JF, Horvath C (1983) Movement of components in reversed-phase chromatography I. Mobile phase space with multi-component eluents. J Chomatogr 282: 211–228.

Melander W, Horvath C (1980) High-Performance Liquid Chromatography. Advances and Perspertives, Volume 2 Academic Press: New York, p 268.

Meyer R, Jira Th (2007) Calixarene HPLC phases – applications. Curr Anal Chem 3: 161–170.

Montgomery ME, Green MAJ, Wirth MJ (1992) Orientational dynamics of a hydrophobic guest in a chromatographic stationary phase: effect of wetting by alcohol. Anal Chem 64: 1170–1175.

Mountain RD, Thirumalai D (2003) Molecular dynamics simulations of end-to-end contact formation in hydrocarbon chains in water and aqueous urea solution. J Am Chem Soc 125: 1950–1957.

Sander LC, Callis JB, Field LR (1983) Fourier transform infrared spectrometric determination of alkyl chain conformation on chemically bonded reverses-phase liquid chromatography packings. Anal Chem 55: 1068–1075.

Sander LC, Lippa KA, Wise SA (2005) Order and disorder in alkyl stationary phase. Anal Bioanal Chem 382: 646–668.

Sander LC, Wiese SA (1993) Shape selectivity in reversed-phase liquid chromatography for the separation of planar and non-planar solutes. J Chromatogr A 656: 335–351.

Sander LC, Wise J (2001) The influence of column temperature on selectivity in liquid chromatography for shape-constrained solutes. J Sep Sci 24: 910–920.

Sandrine Dalbin S, Maurin G, Nogueira RP, Persello J, Pommier N (2005) Silica-based coating for corrosion protection of electrogalvanized steel. Surface Coat Technol 194: 363–371.

Sentell KB, Dorsey JG (1988) On the calculation of the stationary phase volume in reversed phase chromatography. J Liq Chromatogr 11: 1875–1885.

Smith RJ, Nieass CS, Wainwright WS (1986) Review of methods for the determination of hold-up volume in modern liquid chromatography. J Liq Chromatogr 9: 1387–1430.

Singh S, Wegmann J, Müller AK (2002) Variable temperature FT IR studies of n-alkyl modified silica gels. J Phys Chem B 106: 878–888.

Srinivasan G, Meyer Ch, Welsch N, Albert K, Müller K (2006) Influence of synthetic routes on the conformational order and mobility of C18 and C30 stationary phases. J Chromatogr A 1113: 45–54.

Sun L, Siepmann I, Schure MR (2006) Conformation and solvation structure for an isolated n-octadecane chain in water, methanol and their mixtures. J Phys Chem B 110: 10519–10525.

Tessier PM, Lenhoff AM, Sandler SI (2002) Rapid measurement of protein osmotic second virial coefficients by self-interaction chromatography. Biophys J 82: 1620–1631.

Tolls J, van Dijk J, Verbruggen EJM, Hermens JLM, Loeprecht B, Schürmann G (2002) Aqueous solubility-molecular size relationships – A mechanistic case study using C10- to C19-alkanes. J Phys Chem A 106 : 2760–2765.

Wick CD, Siepmann JI, Schure MR (2004) Simulation studies on the effects of mobile-phase modification on partitioning in liquid chromatography. Anal Chem 76: 2886–2892.

Wong AL, Hunnicutt ML, Harris JM (1991) Structure of alkylated silica surfaces: quenching of fluorescence from covalently bound pyrene. Anal Chem 63, 1076–1081.

Ying PT, Dorsey JG, Dill KA (1989) Retention mechanisms of reversed-phase liquid chromatography: determination of solute-solvent interaction free energies. Anal Chem 61: 2540–2546.