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Development of the Tracer-Pulse Method for Adsorption Studies of Analyte Mixtures in Liquid Chromatography Utilizing Mass Spectrometric Detection

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The tracer-pulse method provides the real adsorption data points directly from simple, straightforward calculations and is therefore a superior method for multicomponent adsorption isotherm determination in HPLC. Only one important problem has restricted its use so far: the tracer peaks are invisible using any conventional detection principle. We present a solution to this problem with an approach with a firm base in analytical chemistry, utilizing stable isotopes and mass spectrometric detection. The new approach was used for the determination of binary adsorption isotherms, and a systematic investigation was made of its main sources of error. With this modification, the tracer method can be a prime choice for future characterizations of multicomponent separation systems and of competitive drug binding studies.

Adsorption isotherms describe the partitioning of solutes between mobile and stationary phases at a given constant temperature and are important for a deeper characterization of both analytical and preparative separation systems.[1,2] An analytical example is the detailed characterization of drug−protein interactions using immobilized protein stationary phases.[3] Such systems often contain complex multisite interactions with different affinities and capacities and their adsorption isotherms must be determined in a wide concentration range.[4] However, today there exists no good method for determination of the raw adsorption isotherm data (i.e., the real data points) from a mixture; most methods only give the best parameter estimates.

A classical method for determination of the adsorption isotherms is frontal analysis (FA), which is generally recognized in a single component system to be the most accurate method. FA has been used for binary and ternary mixtures.[5] A prerequisite is that the composition of the intermediate plateaus can be determined, which means that a fractionation and reinjection procedure must be followed for systems with more than two components.[1] A disadvantage is that high-efficiency columns are required for ternary mixtures, to counteract the erosion of the intermediate plateaus by kinetic effects.[5]

The tracer-pulse method (TP) and the perturbation peak method (PP) belong to the so-called pulse methods.[6] If a small excess of molecules is injected into a column equilibrated with an eluent containing the same solute molecules (a concentration plateau), one single peak will appear in the chromatogram. In reality, a total of three zones are created, one of them being the displaced plateau molecules (i.e., the zone visualized as a peak) and another later eluting zone being the injected molecules (tracer peak). The latter zone will not be visualized as a peak since it has a combined elution with the third zone, a deficiency zone of the plateau molecules. This is the simplest case, taking place in a one component system with a convex adsorption isotherm. For a system showing a concave adsorption isotherm, the invisible tracer peak should instead elute faster than the perturbation peak. The tracer peak is impossible to detect unless the sample molecules are labeled somehow so that they can be distinguished from the plateau molecules. The perturbation peaks can be used for adsorption isotherm determinations because the velocity is related to the tangential slope of the adsorption isotherm at the actual concentration plateau whereas the velocity of the tracer peak is governed by the corresponding chord. However, as will be seen below, the latter relation (TP) is much simpler and straightforward for calculation of adsorption data than the former one (PP), especially for competitive adsorption.

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Both pulse methods were suggested in the early 60s when gas chromatography started to be recognized not only as an analytical tool but also as a powerful method for measuring thermodynamic properties.[6] The GC community focused on the TP because of its more elegant and simple theoretical solution and because of the great variety of on-line detectors available allowing for detection of labeled molecules.[7] The PP was considered a "less accurate" differentiate method[5] and fell into oblivion. The first experimental applications of the TP used radioactive isotopes,[8,9] but difficulties such as the handling of radioactive solutes in the gas phase hindered the method to become widespread. In the next decade, when mass spectrometry became a standard detector for GC, TP was used with stable isotopes detected with a quadrapole instrument.[10,11] However, the new application was introduced at a time when the interest for measuring thermodynamic quantities in GC had declined. During recent years in LC, the PP theory was extended to encompass the multicomponent case and also validated for these adsorption isotherm determinations.[12,13]

Recently, fundamental studies has been made of the TP in HPLC.[14−17] The hidden events in the column were visualized using two different experimental strategies: (i) a radioactive labeled approach and (ii) a method based on the use of two pure enantiomers in a nonchiral separation system.[14] A systematic investigation of a similar phenomenon was made for frontal analysis,[15] and also very strange deformations of overloaded tracer zones[16] were visualized and systematically investigated. A tedious chiral approach of TP was applied for determination of multicomponent adsorption isotherms.[17]

The TP is the superior method for studies of competitive adsorption but it has one serious problem, which has restricted its widespread use in HPLC, how can we selectively detect only a few injected molecules in a large population of identical ones? A smart mass spectrometric solution might be a way to solve the problem but has not been tried for the TP in LC yet. However, time-of-flight secondary ion mass spectrometry (TOF-SIMS) has been used for quantitative surface analysis and direct measurement of adsorption isotherms.[18,19] Mass spectrometry has also been combined with frontal analysis for selective and rapid screening of important inhibitor components in crude extracts.[20,21]

The aim of this study is threefold: (i) to develop a method for implementation of mass spectrometric detection of stable isotopes in the TP for LC, (ii) to systematically investigate the main sources of error of the developed method, and (iii) to apply the method for a multicomponent system.

THEORY

For a more thorough review of the theory involved, we refer to previous work.[16,17] In this study the competitive Langmuir adsorption isotherm will be used:

$$q_i = \frac{a_i C_i}{1 + \sum_i b_i C_i} \quad i = 1,2,...,n \tag{1}$$

where q_i and C_i are the stationary and mobile phase concentrations of component i, a_i is the distribution coefficient, and b_i is the association equilibrium constant.

Perturbation peaks, measured in the PP, originate from the disturbance of the established solute equilibrium in the column. In a two-component system, two perturbation peaks will be present and the retention time of these peaks are

$$t_R = \frac{t_0}{2}\left[2 + F\left(\frac{\partial q_1}{\partial C_1} + \frac{\partial q_2}{\partial C_2}\right) \pm F\sqrt{\left(\frac{\partial q_1}{\partial C_1} - \frac{\partial q_2}{\partial C_2}\right)^2 + 4\frac{\partial q_1}{\partial C_2}\frac{\partial q_2}{\partial C_1}}\right]_{C=C_0} \tag{2}$$

where t_0 is the column hold-up time, F is the volumetric phase ratio, C_0 is the eluent solute concentration and $q_i(C_1,C_2)$ is a competitive adsorption isotherm. It is important to note that it is not possible to identify the perturbation peaks in a two-component system, because both components contribute to retention times and areas of each perturbation peak.

Tracer peaks consist of, in contrast to perturbation peaks, the actual injected molecules and can thus be identified also in a multicomponent system. In the TP, the injected solutes are labeled so that each tracer peak can be followed by a selective detector. In a two-component system, two tracer peaks will be present with retention times according to

$$t_{R,i} = t_0\left(1 + F\frac{q_i(C_1, C_2)}{C_i}\right)_{C=C_0} \tag{3}$$

EXPERIMENTAL SECTION

Apparatus. The chromatographic system was an Agilent 1100 from Agilent Technology (Palo Alto, CA) consisting of binary pumps, autosampler, and a diode-array UV detector. The column, an Eclipse XDB-C8 (4.6 mm × 150 mm, 5 μm) from Agilent Technologies (Palo Alto, CA), was placed in a laboratory-assembled column jacket and its temperature was controlled at 29.0 °C using a LAUDA type B circulating water bath (Königshofen, Germany). All tubings in the chromatographic system before the first flow-split was 0.13 mm PEEK, and the flow rate was 0.70 mL/min. An API III plus triple quadrupole mass spectrometer from PE-Sciex (Concord, ON, Canada) equipped with an articulated IonSpray (pneumatically assisted electrospray, ESI) interface was used.

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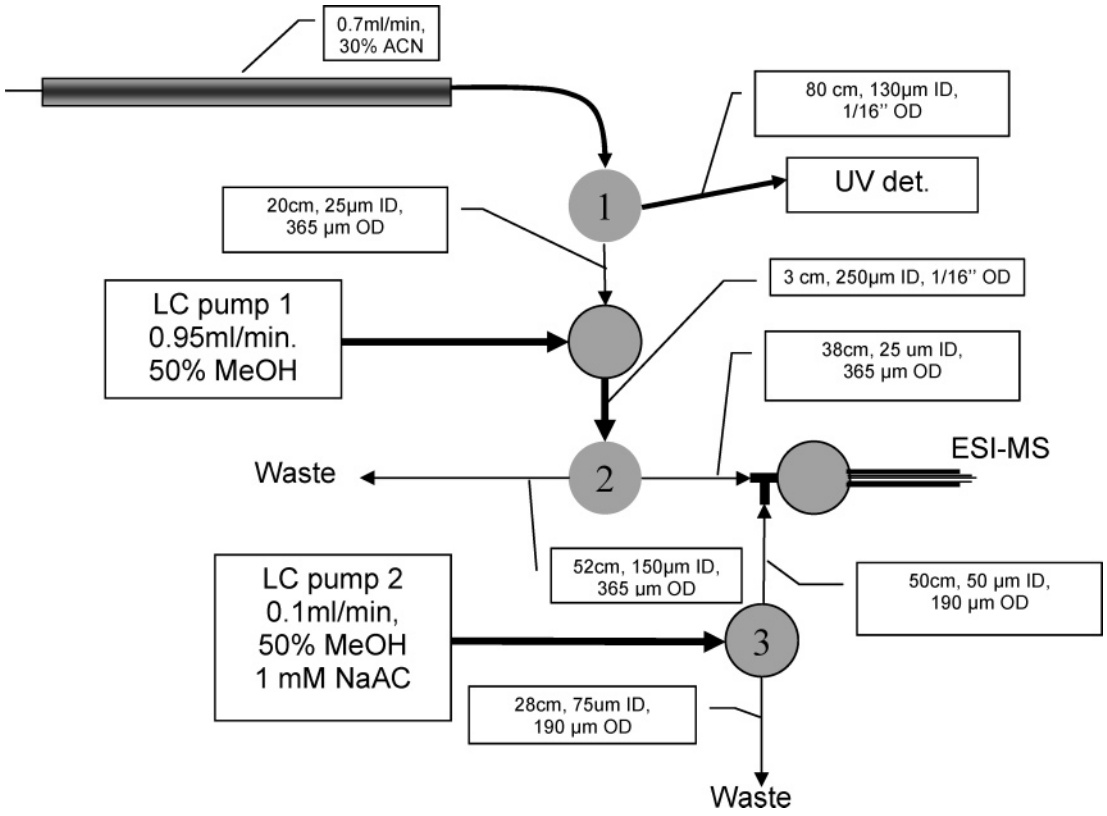

Figure 1. Illustration of the flow-splits and the makeup flows between the column and the mass spectrometer. Flow-split: (1) splits the flow 1:350 times, (2) 1:950 times, and (3) 1:10 times.

Chemicals. Acetonitrile, methanol (CHROMASOLV quality), sodium acetate (>99%), uracil (>99%), and methyl- and ethyl mandelate (>98%) were bought from Sigma-Aldrich. The water used was from a Milli-Q water purification system ZMQS 5000Y from Millipore (Molsheim, France).

Solute Synthesis. All chemicals were used as received except for methyl mandelate, which was recrystallized from a mixture of *n*-pentane/Et_2O (9/1), and ethyl mandelate, which was purified on a silica column using freshly distilled *n*-pentane/ethyl acetate (7/3) as an eluent. Toluene was dried over sodium metal with a sodium benzophenone ketyl indicator prior to use. The reactions were performed under an N_2 atmosphere. The deuterium labeled methyl mandelate (MM*) and ethyl mandelate (EM*) were synthesized by a procedure modified from that of Toshikatsu et al.[22] ^{1}H NMR spectra were recorded at 500 MHz. Chemical shifts are reported in ppm, relative to the residual solvent peak $CDCl_3$ (7.26 ppm). ^{13}C NMR spectra were recorded at 125 MHz, and chemical shifts were reported relative to the solvent peak of $CDCl_3$ (77.0 ppm). Mass spectra were measured at 70 eV (EI) and are reported as m/z (relative intensity, %). Exact mass measurements were made with an ESI-TOF mass spectrometer (Agilent Technologies series 1100 LC−MSD TOF mass spectrometer). IR spectra were recorded on a FT-IR with an ATR accessory, and signals are reported as $\tilde{\nu}$ (cm^{-1}, relative intensity).

Synthesis of Methyl-d_3-mandelate (MM).* To a stirring solution of mandelic acid (0.50 g, 3.29 mmol) in a mixture of toluene (5 mL) and methanol-d_4 (1 mL), boric acid (10 mol %) was added. The reaction was stirred at ambient temperature for 24 h. The reaction mixture was then diluted with Et_2O (10 mL), washed with $NaHCO_3$ (aq) (3 × 5 mL) and subsequently with brine (1 × 5 mL), and dried over $MgSO_4$. The organic phase was filtered, and the solvent was removed under reduced pressure giving 0.51 g, 91% of MM* as transparent crystals.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.33−7.43 (m, 5H), 5.18 (s, 1H), 3.55 (br s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.1, 138.2, 128.6, 126.6, 72.9, 52.2 (h, $^1J_{CD}$ = 22.3 Hz, intensity ratio 1:3:6:7:6:3:1). FT-IR (neat): 3435, 3035, 2078, 1736, 1213, 1066. EI-MS: 169.1 (33.1), 152.1 (54.4), 107.1 (47.5), 77.1 (100). Exact mass (ESI) m/z calcd for $C_9H_7D_3O_3Na$ [M + Na^+] = 192.0716, found: 192.0682.

Synthesis of Ethyl-d_5-mandelate (EM).* The procedure was exactly as above except that 1.97 mmol of mandelic acid was used, and methanol-d_4 was exchanged to ethanol-d_6 (0.5 mL). This resulted in transparent crystals (0.32 g, 87%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.35−7.47 (5H), 5.18−5.20 (m, 1H), 3.60−3.62 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.7, 138.4, 128.3, 126.5, 72.9, 61.3 (q, $^1J_{CD}$ = 23.6 Hz, intensity ratio 1:2:3:2:1), 12.9 (h, $^1J_{CD}$ = 19.4 Hz, intensity ratio 1:3:6:7:6:3:1). FT-IR (neat): 3437, 2914, 2245, 1725, 1184, 1059. EI-MS: 186.0 (17.3), 168.1 (29.5), 107.1 (48.7), 77.1 (100). Exact mass (ESI-MS) m/z calcd for $C_{10}H_7D_5O_3Na$ [M + Na^+] = 208.0998, found: 208.0955.

Chromatographic Procedures. The eluent was 30/70 (v/v) acetonitrile/water. The column hold-up time was estimated to 2.00 min with a RSD of 0.04% (n = 5) by injecting uracil which is generally recognized to be slightly retained. The lag time between the UV detector and the mass spectrometer was determined by injections of 20 µL of 7.5 mM of MM and EM to 0.365 min with

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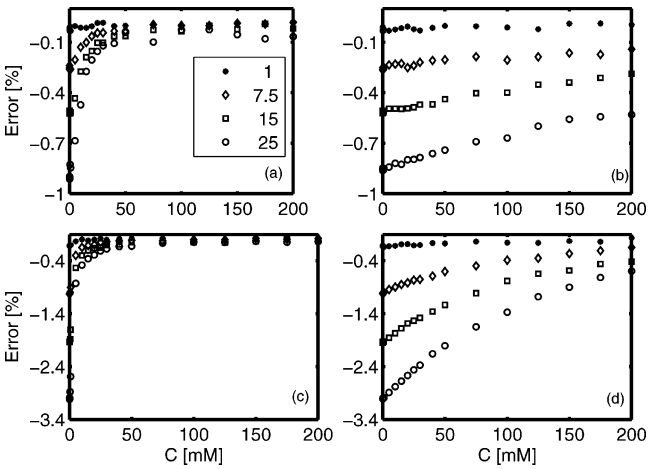

Figure 2. The relative error in the retention time versus eluent concentration for the tracer peak (a, c) and the perturbation peak (b, d), respectively, calculated for a 20 μL injection of 1, 7.5, 15, and 25 mM excesses (see legend). Two different Langmuir adsorption isotherms were used (a, b) $a = 4.59$ and $b = 2.31$ M^{-1} and (c, d) $a = 4.59$ and $b = 9.17$ M^{-1}. The number of theoretical plates was 7030.

Table 1. The Error on the Estimated Adsorption Isotherm Parameters Due to the Injection Volume[a]

	tracer-pulse method				perturbation peak method			
	parameters		error		parameters		error	
C_{inj} [mM]	a	b [1/M]	a [%]	b [%]	a	b [1/M]	a [%]	b [%]
1	4.585	2.309	−0.02	−0.11	4.584	2.310	−0.03	−0.07
7.5	4.583	2.305	−0.06	−0.28	4.571	2.308	−0.32	−0.14
15	4.580	2.303	−0.11	−0.35	4.555	2.304	−0.66	−0.30
25	4.577	2.298	−0.19	−0.57	4.535	2.301	−1.10	−0.44
true	4.586	2.311			4.586	2.311		
1	4.585	9.167	0.00	−0.05	4.578	9.164	−0.16	−0.08
7.5	4.580	9.149	−0.12	−0.25	4.526	9.122	−1.30	−0.53
15	4.576	9.145	−0.21	−0.28	4.471	9.092	−2.49	−0.86
25	4.570	9.128	−0.34	−0.47	4.408	9.066	−3.88	−1.15
true	4.586	9.171			4.586	9.171		

[a] The error is defined as (determined-true)/true adsorption isotherm and is expressed as percentage. All conditions as in Figure 2.

RSD of 6.4% ($n = 6$). The labeling effect was determined by injections of 7.5 mM labeled and unlabeled MM and EM, to −0.06 min with a RSD of 14% ($n = 3$) and to −0.19 min with a RSD of 6.2% ($n = 3$), respectively. The column efficiency was determined to 7030 with an RSD of 1.8% ($n = 4$). The UV detector was set at 260 nm for low and medium solute concentrations and 275 nm for high solute concentrations.

Samples were analyzed in positive ESI mode with a spray and orifice potential of 3500 and 50 V, respectively. The nebulization gas pressure (N_2, boil-off from liquid nitrogen) was 40 psi. Data were acquired with a dwell time of 100 ms in SIM mode. The monitored ions, corresponding to the sodiated molecules, were facilitated by the addition of 10 μL/min, (50% methanol) of a sheath-flow containing 1 mM sodium acetate, where the monoisotopic and isotopic peaks were for MM 189, 190; MM* 192; EM 203, 204; and EM* 208.

The determination of adsorption isotherms were conducted using three different stock solutions each (low, mid, and high concentration) of single MM respective EM and of their 1:1 mixture. The stock solutions were 1.02, 10.2, and 205 mM MM; 0.625, 6.25, and 125 mM EM; and 0.503 + 0.503, 5.03 + 5.03, and 101 + 101 mM mixture.

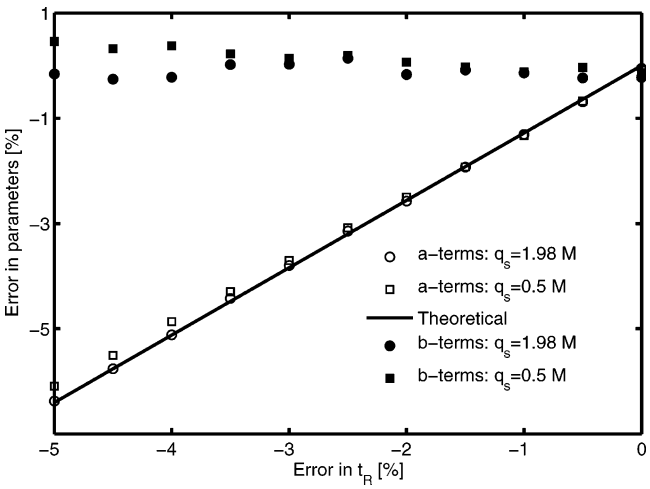

Figure 3. The relative error in the adsorption isotherm is plotted against the labeling effect as the error in the retention time compared to the unlabeled substance. Unfilled symbols are for the a term and filled symbols for the b term, and the line is the calculated error due to the labeling effect. Two different Langmuir adsorption isotherms were used: $a = 4.59$ and $b = 2.31$ M^{-1} (circles) and $a = 4.59$ and $b = 9.17$ M^{-1} (squares).

The plateau concentrations were constructed by connecting the outlets of the flows of pure eluent (pump 1) and a stock solution (pump 2) through a low-dead-volume PEEK tee before entering the column. The percentage stock solutions used were 30, 50, 70, and 100% for low and mid stock solutions and 20, 40, 60, and 100% for high stock solutions. Determination of the adsorption isotherms were conducted by injecting 20 μL of 7.5 mM labeled solute. The labeled solutes were dissolved in pure eluent except when the high concentration stock solutions were analyzed. Then it was dissolved in diluted high stock according to 50% stock at the 20 and 40% plateaus, 75% stock at the 60% plateau, and 100% stock at the 100% plateau.

All simulations were done using the equilibrium-dispersive model, solved by using a modified Rouchon method.[23] Injection profiles were measured experimentally and were used as boundary conditions in the simulations. Detector calibration was performed by converting response to concentration by a third degree polynomial function.

RESULT AND DISCUSSION

Implementation of Mass Spectrometric Detection. The TP has one major practical problem; the tracer zone cannot be visualized as a peak by conventional detection principles. We have previously used enantiomers and radioactive labeled compounds to visualize the tracer peaks and thus to determine adsorption isotherms with the TP.[16] However, the enantiomeric approach is very time-consuming and tedious and also not general (chiral systems cannot be characterized). The radioactive labeling is more general, but the detector is not as selective as the mass spectrometer and the labeled compound could break down due

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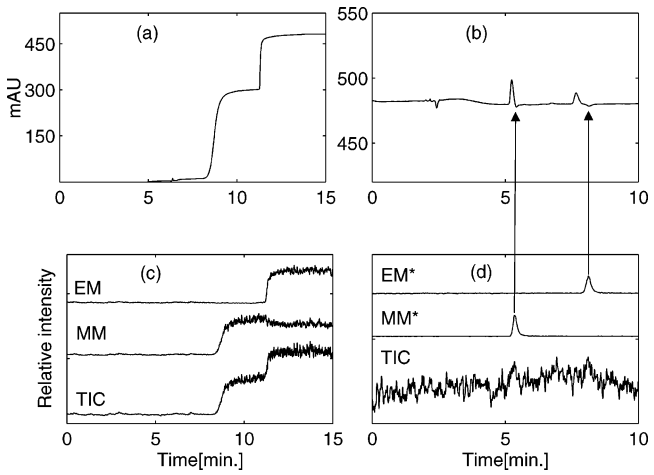

Figure 4. Parts a and c show the breakthrough of the eluent 40/40 mM of MM/EM and parts b and d show the elution of a 20 μL injection of 7.5/7.5/50/50 mM of MM*/EM*/MM/EM on a concentration plateau of 40/40 mM of MM/EM. The top row (a, b) is the UV-detector signal, and the bottom row (c, d) shows the MS signals. Observe that the MS baselines have been moved for easier visualization. TIC is the total ion count.

to self-radiolysis. In addition, special care must be considered for handling and disposal of radioactive material.

In this study, we developed a TP method based on using stable isotopes measured with mass spectrometry (MS) to determine adsorption isotherms of mixtures. The new approach has several advantages compared to the previous ones: less tedious, more general, and no special care need to be taken during handling of the solutes. Still some problems needed to be considered: saturation of the detector, MS-compatibility of the column effluent, and long-term stability. The origin of these problems is that a wide range in concentration, from very low to very high, is necessary for proper isotherm determination when the purpose is to make a complete census of all interactions in the separation system. These problems were solved by dilution of the highly concentrated column effluent in a stepwise manner, see Figure 1. The first split reduced the flow to the mass spectrometer line down to around 2 μL/min while the rest of the flow is introduced into the UV detector. After the first split, the effluent is diluted 476 times by introducing 0.95 mL/min 50% (v/v) methanol/water. After the dilution, the flow is once again reduced by a factor of around 950 down to around 1 μL/min. Finally, the effluent is diluted 11 times by introducing 10 μL/min sheath-flow containing 1 mM sodium acetate in 50% (v/v) methanol/water (sodium is introduced because MM and EM is detected as sodium adducts). Thus, the column effluent is totally diluted more than 5000 times which allows us to use large solute concentrations in the eluent while still operating in the linear dynamic range of the detector and an addition decrease in ion suppression in the ion source. Furthermore, the mass entering the ion source is reduced more than 330 000 times, allowing us to run the experiments for longer time without needing to clean the mass spectrometer system. Finally we could optimize the separation and use high concentrations of nonvolatile solutes and buffer components that are not MS-compatible, because the compatibility could be solved postcolumn using the splits.

The disadvantage with the split is that it forces us to inject a large excess of labeled solutes on low-plateau concentrations, this was due to the lost sensitivity as the column effluent was diluted. We therefore made a systematic investigation of the error due to a large excess of the injected tracer (below). Another source of error that we investigated systematically was the labeling effect; i.e., the tracer does not have exactly the same properties as the unlabeled molecule.

Error Due to the Excess Injected. The PP theory assume that the injected sample that create the equilibrium disturbance gives a negligible concentration deviation compared to the plateau concentration, so that dq/dC does not change because of the injected concentration deviation (cf. eq 2). In practice this is not possible, and the PP experiences a more or less large error due to the injected excess;[13] below we will investigate if there exists a similar error for the TP. In the TP, the injected sample also contains a tracer, i.e., labeled molecules with identical properties as the unlabeled ones. The chord of the adsorption isotherm should not change due to the injected concentration deviation (see eq 3). We previously showed qualitatively that if a large excess is injected, the tracer peak will be deformed and have a nonrepresentative retention time.[16] It was also shown that if no excess is injected, i.e., the total sample concentration (labeled + unlabeled) is identical to the plateau concentration, no perturbation peak will appear and the tracer signal has an ideal and identical retention time independent of size of the tracer peak.[16] The preparation of ideal tracer samples, one for each plateau, is however a very tedious procedure, so in practice small deviation to the eluent would be preferred. In the present study we performed a more quantitative analysis in order to investigate how the size of the excess (concentration in mobile phase + excess) affects the determination of adsorption isotherms and the retention time for the perturbation and tracer peaks. For this purpose a series of simulations were done with 20 μL injections containing an excess of 1, 7.5, 15, and 25 mM on concentration plateaus between 0.01 and 200 mM. In the investigation, two different Langmuir adsorption isotherms were used with $a = 4.59$ and $b = 2.31$ M^{-1} and $a = 4.59$ and $b = 9.17$ M^{-1}, corresponding to monolayer saturation capacity of 1.98 and 0.5 M, respectively.

In Figure 2, an error analysis is performed for the tracer (a and c) and for the perturbation peak (b and d) for an adsorption isotherm with $q_s = 1.98$ M (a and b) and with $q_s = 0.5$ M (c and d). The relative retention time error $((t_R - t_{R,ideal})/t_{R,ideal}$ where $t_{R,ideal}$, is calculated with eqs 2 or 3) was plotted against the bulk eluent concentration. Observe that in these cases (convex type I adsorption isotherms with injected excesses), the retention times from the tracer and perturbation peak is always shorter compared to the true retention time with infinitesimal excess. The opposite would be true for type III (concave adsorption isotherm), and for other types of adsorption isotherms (e.g., S-shaped) the trend would be eluent concentration dependent. If we instead inject a deficiency, the opposite trend would be noted for all different types of adsorption isotherms.

The relative retention time error for both the tracer peak in Figure 2 (a and c) and the perturbation peak (b and d) increases with increasing injected excess. However, as the plateau concentration increases, the error for the tracer peak declines rapidly giving a much more favorable situation for the TP as compared to the PP. We can also see that a lower monolayer capacity gives a larger error for both methods; this is due to the larger loading

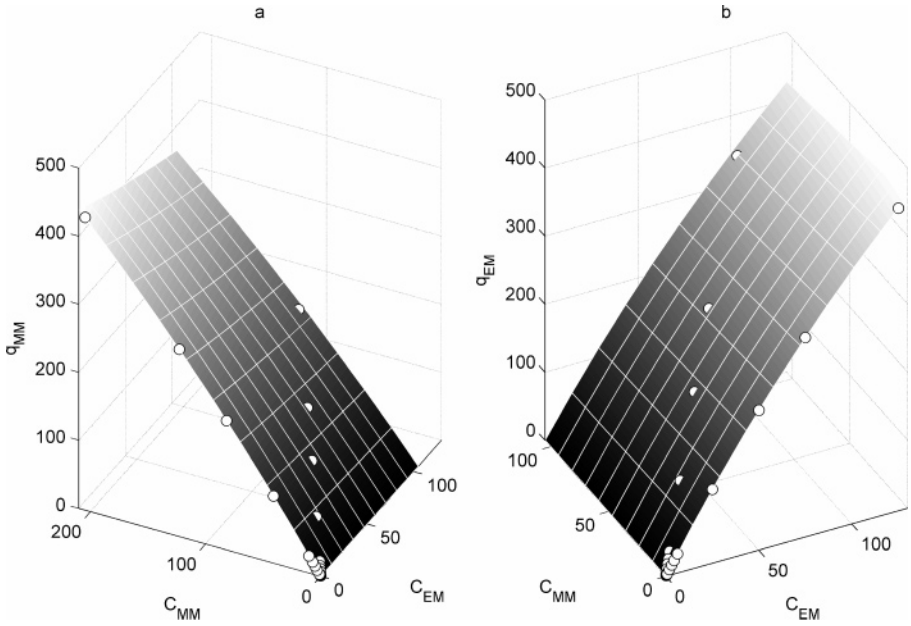

Figure 5. The adsorption isotherm data and the fitted competitive Langmuir model. The stationary phase concentration of (a) MM and (b) EM is plotted versus the mobile phase concentrations. Symbols are experimental data.

factor and thus degree of nonlinearity experienced at the plateau concentration. The reason for the similar error for the TP and PP at low plateau concentrations is that the tracer zone has a more or less combined elution with the perturbation peak. The error decreases drastically for the TP with increasing concentration in the plateau because the tracer peak propagates for a shorter period of time in the perturbation zone and is less affected by the concentration gradient created by the perturbation zone which deforms the tracer zone.[16] The higher the eluent concentration, the larger is the difference in velocity between the zones and the faster does the tracer zone leave the perturbation zone.

Several different practical problems exist when determining adsorption isotherms with the PP. The first is to detect the peak; here we need to inject a finite plateau concentration deviation, leading to thermodynamic tailing. Gritti et al. reduced the error in the determined retention time for the perturbation peak by injecting a positive and a negative plateau deviation following each other, so that the peaks had equal area and took the average of the retention times from these peaks.[24] Even if the error is reduced considerably, this approach will not totally compensate for the error because that would require that the tailing of the positive and negative peak is identical but opposite to each other. This is not necessarily true depending on the particular curvature of the adsorption isotherm (which is unknown as the measure takes place). Usually the detector sensitivity decreases with increasing plateau concentration, so that larger absolute deviations compared to the plateau concentration must be injected. For the TP, the error decreases drastically with increasing plateau concentration and is not really affected by increased injection excess at high plateau concentration, which is a great advantage as compared to the PP. Cavazzini et al. used the perturbation peak method to determine the adsorption of MeOH in a straight phase system.[25] The authors note that PP gave large errors in the low-concentration region, the solution was to use large perturbations, and use the inverse method to find adsorption isotherm parameters to estimate the retention time at extremely low plateau concentrations. However, it should be mentioned that smaller excess could be used for PP and TP if we do not need to split the column effluent, resulting in smaller errors.

Lindholm et al. have studied the relative perturbation peak retention error but reached slightly different results.[13] The authors investigated how the error changes with increasing eluent concentration for blank injections, 100% and 50% excess compared to the eluent. They found that the error in the linear range of the adsorption isotherm is very small, has a maximum in the weakly nonlinear range, and decreases slowly at higher concentration. In the present study a more pragmatic approach is taken: we instead use constant (not relative) excess and no such maximum was found (see Figure 2). Our results are also in line with the recent observation of Cavazzini et al. that the PP has large errors in the low-concentration region.[25]

In Table 1, the error in the determined adsorption isotherm parameters are presented. The error for the TP is generally much lower as compared to the PP. The TP delivers good predictions of the *a* term with an error below 0.4%. For the PP, the error is up to around 10 times larger. The error in the *b* term is similar for both methods for the high-capacity column, but as the capacity of the column decreases, the error in the *b* term increases and the PP delivers approximately twice as large errors compared to the TP.

The maximum error experienced for the tracer peak experiments in this study corresponded approximately to the situation with an excess injected of 7.5 mM at low-concentration plateaus.

Error Due to the Labeling Effect. The labeled molecules (MM*, EM*) are somewhat less retained than the unlabeled ones (MM, EM), and this might affect the determined adsorption isotherm. MM* eluted 0.06 min before MM, and EM* eluted 0.19

(24) Gritti, F.; Piatkowski, W.; Guiochon, G. *J. Chromatogr., A* **2003**, *983*, 51−71.

(25) Cavazzini, A.; Nadalini, G.; Malanchin, V.; Costa, V.; Dondi, F.; Gasparrini, F. *Anal. Chem.* **2007**, *79*, 3802−3809.

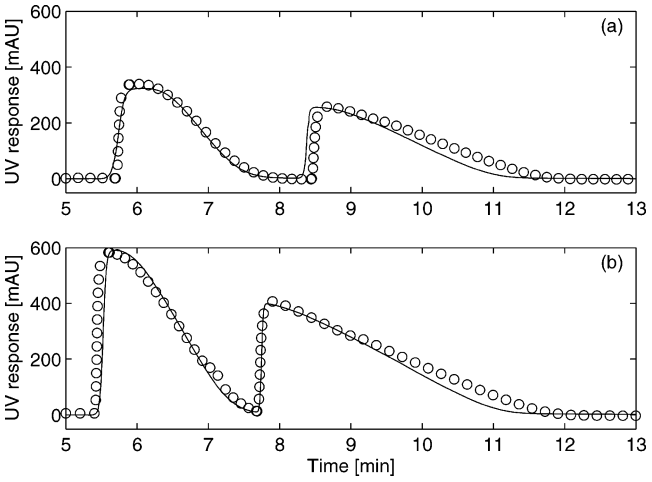

Figure 6. Predicted band profiles for the 900 μL injection of (a) 50 mM of EM and MM and (b) 100 mM of MM and EM. The Langmuir parameters used were $a = 2.49$, $b = 0.906$ M^{-1} (MM) and $a = 4.59$, $b = 2.31$ M^{-1} (EM). The solid lines are calculated profiles, and the symbols are experimental data.

min before the EM, which is 0.95% and 1.9% of the total retention time of MM and EM, respectively. The labeling effect on EM will therefore have a larger effect on the adsorption isotherm as compared to MM. To estimate how large of an error a labeling effect will have on the determined adsorption isotherm, simulations of 20 μL excess injections of 7.5 mM on plateau concentrations of eluents between 0.01 and 200 mM were performed by changing the distribution coefficient to the desired retention time and changing the equilibrium constant in such a way so that the column saturation capacity ($q_s = a/b$) was constant. The Langmuir models with parameter sets defined above were used; the labeling error was allowed to vary between 0 and 5%. The acquired data were fitted to the Langmuir model and are presented in Figure 3.

In Figure 3, one can see that the fitted distribution coefficient is underestimated and that the underestimation increases proportionally with increasing labeling effect; in fact the constant coincides more or less with the assumed labeling effect (theoretical line in Figure 3). At a 2% error in the retention time, the distribution coefficient is decreased by 2.5%. The error in the equilibrium constant (b) is small and does not change with increasing labeling error. The two different Langmuir adsorption isotherms described above were used $q_s = 1.98$ M (circles) and $q_s = 0.5$ M (squares) but no clear difference using these two capacity values could be recognized (cf. Figure 3). The determined labeling effect is in line with a recently presented general indirect detection theory, where the retention time of the solute is described by the probe's equilibrium constant (in this case, the plateau molecules) and the solute's distribution coefficient (in this case, the tracer).[26]

The labeling effect could be decreased by substituting less hydrogen to deuterium or use ^{13}C instead, but it is recommended to have at least three mass unit differences between the stable isotopes and the unlabeled compound to be able to separate them properly in the mass spectrometer.

It is difficult to find similar error data in the literature as a comparison, i.e., studies on the systematic error of raw data. We could only find another paper dealing with this type of error, it was when using the frontal analysis method in the staircase mode where a serious source of error was found in the determination of the volume between the T-connector (where the two pumps meet) and the column.[13] Two good but different methods gave results that varied more than 5% which was not acceptable (see Figure 4 in ref 13). Only one of the methods gave a value which coincided with the PP method, and this value was therefore assumed to be the correct one.

Experimental Determination of Competitive Adsorption Isotherms. Because of the different mass of unlabeled and labeled MM and EM, they could be detected by MS. The UV detector gives the sum of all events in the chromatographic process. Figure 4a,c presents the breakthrough curve of 40/40 mM of MM/EM. In parts b and d a 20 μL injection of 7.5/7.5/50/50 mM of MM*/EM*/MM/EM is injected on the established concentration plateau of 40/40 mM of MM/EM. Parts a and b show the UV signal, and parts c and d show the MS signals.

In the UV track, two fronts can be seen with an intermediate concentration plateau in between. The single ion monitoring (SIM) tracks reveal that MM is displaced by the EM, and the intermediate plateau has a higher concentration as compared to MM in the eluent. The displacement effect is a sign of competition inside the column. As expected, the perturbation peaks elute before the mass peaks (cf. Figure 4b,d). The perturbation and tracer peaks cannot be distinguished in the total ion count (TIC), due to the high ion current. However, in SIM mode the tracer peaks are easily detected. All the MS signals were slightly noise reduced due to the high-background noise.

Adsorption isotherm parameters were fitted to the tracer-pulse retention data. Nonlinear regression was performed using the lsqnonlin algorithm as implemented in Matlab (MathWorks Inc., Nattick, MA). A global solution was sought by repeating the fitting 1000 times with random initial guesses spread over the whole feasible solution space. The data fitted well to the Langmuir adsorption isotherm (eq 1). Other models were tried as well, but no statistically significant improvement was obtained. The Langmuir parameters were as follows: for MM $a = 2.49$, $b = 0.906$ M^{-1} and for EM $a = 4.59$, $b = 2.31$ M^{-1}. This results in a saturation capacity of 2.75 and 1.98 M, respectively. The fitted competitive 3D-adsorption isotherms are plotted with the data in Figure 5: it can be observed that the degree of nonlinearity is moderate even at 100−200 mM concentrations of MM and EM.

Computer simulation band profiles were compared to experimental chromatograms. Figure 6 shows the simulated and experimental chromatograms corresponding to 900 μL injections of (a) 50/50 mM of MM/EM and (b) 100 /100 mM of MM/EM. The agreement is very convincing. A small deviation can be seen at the diffusive rear of EM; the simulated signal reaches baseline earlier than does the experimental signal. This is probably due to an underestimation of the EM a term caused by the labeled effect on the tracer.

CONCLUSION

The tracer-pulse method is a very good method for measuring competitive adsorption isotherms since it delivers the real data points on the adsorption isotherm (i.e., not only the parameter estimates). Moreover, the TP theory is simple and straightforward for any number of compounds in contrast to other methods for

(26) Forssén, P.; Fornstedt, T. *J. Chromatogr., A* **2006**, *1126*, 268−275.

n-component determinations such as the inverse method and the perturbation method. In addition, in a multicomponent situation, the tracer peaks can always be identified in contrast to perturbation peaks. However, one important problem has restricted its widespread use in HPLC: how to practically visualize the tracer peaks.

We here present a solution to this problem, with a firm base in analytical chemistry, by the implementation of stable isotopes detected with mass spectrometry. This approach was used for determining the competitive adsorption isotherms of a mixture of methyl- and ethyl mandelate. The method development involved the solving of many practical problems associated with highly concentrated effluents and MS, by designing triple flow splits in series for reducing the mass by more than 300 000 times and diluting the effluent more than 5000 times before entering the mass spectrometer inlet. Using these splits makes it possible to use concentrated solutions of non MS-compatible mobile phases and solutes, allowing a more general investigation to be conducted.

We systematically investigated the main sources of errors of the method. One important source of error for a relating technique, the perturbation method, is a too large equilibrium disturbance made on the system. We found that the largest errors in the retention times, due to an excess tracer injected, occurred at low-plateau concentration and that the errors decreased very drastically for the tracer peak, as compared to the perturbation peak, with increasing plateau concentration. The error due to the labeling effect was also systematically investigated. Theoretically the tracer pulse method is derived assuming that the tracer should have exactly the same properties as the unlabeled compound; this is, however, seldom the case. We found that the labeling does not affect the determination of the equilibrium constant, but the initial slope of the adsorption isotherm (distribution coefficient) was more or less identical to the assumed distribution coefficient of the labeled molecules.

The model independent raw adsorption isotherm data delivered by the tracer-pulse method gives the opportunity to derive the number of different interactions that take place simultaneously in a complex sample mixture and to calculate the energy involved in each of these different types of interactions (e.g., hydrophobic, electrostatic, polar). This will improve the fundamental understanding of the adsorption processes of mixtures. It will also be possible to make accurate investigations in competitive drug binding studies, with a complete census of all interactions (weak as well as strong interactions).

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