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Review

Ghost peaks in reversed-phase gradient HPLC: a review and update

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Abstract

New examples and sources of ghost peaks in reversed-phase gradient HPLC are described and related to previous publications to give a broad perspective of ghost peak problems. In one new example the ghost peak was found to be due to mixing problems caused by a period of non-delivery of the stronger "solvent B" in a stepped gradient system. In a second example the ghost peak was due to plasticizer contamination of the organic solvent. This paper includes tips and recommendations for the consistent running of ghost-peak-free reversed-phase gradient HPLC.

Keywords: Gradient HPLC; Ghost peaks; System peaks; Chromatographic baselines; Water; Methanol; Acetonitrile; Solvents; TFA; Artifacts

Contents

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1. Introduction

In the highly regulated pharmaceutical industry 'control' is fundamental to all aspects of production and analysis. Reversed-phase gradient HPLC enables the analysis of a mixture of analytes possessing a broad range of polarity and retention characteristics in a single run, and for many applications this offers significant benefits over isocratic methodology. However, gradient HPLC can sometimes be plagued with seemingly random and uncontrollable "ghost peak" problems, particularly if the equipment and materials available are not of very high quality, or if the analyst does not appreciate the sensitive mechanisms involved. When ghost peaks suddenly appear in gradient HPLC chromatograms, the analyst may feel not in control of the analysis and may resort to less efficient isocratic methods that are ultimately more time-consuming and costly. However, if one understands the origins of ghost peaks and how to deal with them, then gradient HPLC can be run consistently and reliably.

Previously used terms in the literature include ghost peaks, artifact (and artefact) peaks [1], system peaks [2], pseudo peaks [3], vacancy peaks [4], eigenpeaks [2,5], induced peaks [6], and spurious peaks [7] and this lack of common nomenclature makes researching the problem quite difficult [1]. "Ghost peak" now appears to be the most frequently used term [1,4,8–11] and will be used throughout this paper.

There are many possible causes why ghost peaks may be observed in gradient HPLC, however almost always, only one common mechanism is responsible for their appearance. This is that UV absorbing organic impurities in the mobile phase are focused into component bands on the column and subsequently eluted later in the gradient when the mobile phase possesses higher eluotropic strength. The potential 'sources' of these mobile phase impurities are wide-ranging. Other causes of ghost peaks include physical or mechanical aspects of mobile phase delivery, sample introduction and stationary phase effects. A chromatogram may contain ghost peaks from a variety of these sources and this can make the overall resolution of ghost peak problems quite difficult.

In the following discussions, the impurity focusing mechanism is illustrated in more detail and some new and previously acknowledged causes are discussed along with tips for the identification and remediation of ghost peak problems.

2. Experimental

2.1. Instrumentation

Three different HPLC systems were used in this work, specific conditions are described in the text. Figs. 2, 5, 7, 9 and 12 were obtained using a Waters 2695 chromatograph and Waters 2487 variable wavelength detector from Waters Corporation (Milford, MA). Figs. 3, 8, 11 and 13 were generated using an HP1100 system from Agilent Technologies Inc. (Wilmington, Delaware) comprising of a degasser (G1322A), quaternary pump (G1311A), autosampler (G1313A) and VWD detector (G1314A). Fig. 4 was obtained using a similar system, but with a binary pump (G1312A) rather than the quaternary pump.

Fig. 10 was obtained using a Waters 2795 and Waters 996 detector and accurate mass measurements were obtained online using a Micromass LC-TOF operating in +ESI mode, with the following settings: capillary voltage = 3000 V, cone voltage = +25 V, desolvation temp. = 250 °C, source temp. = 120 °C. Gas flows (N_2); cone = 45 L/h, desolvation = 356 L/h.

UV spectra were obtained using a UV2-300 UV–vis spectrometer from Unicam Ltd (Cambridge, UK), with a 1 cm quartz sample cell and an empty reference cell.

Zorbax HPLC columns were supplied by Agilent Technologies Inc. (Wilmington, Delaware), Genesis columns were supplied by Jones Chromatography Ltd. (Hengoed, UK), Hichrom columns were supplied by Hichrom Ltd. (Reading, UK).

2.2. Materials

Water for analysis was purified using a USF Elga Prima and Maxima unit supplied by Veolia Water Systems Ltd. (High Wycombe, Bucks, UK). Methanol, acetonitrile and tetrahydrofuran were obtained from various undisclosed UK suppliers. Formic acid (98/100%) was used as supplied by Fisher Scientific (Loughborough, UK). Trifluoroacetic acid (99% grade and spectrophotometric grade) and dioctyl phthalate 99% (as bis-(2-ethylhexyl) phthalate) were used as supplied by Sigma–Aldrich (Gillingham, Dorset, UK).

3. Results and discussion

3.1. Mobile phase contamination and the band compression mechanism

In all chromatography longitudinal diffusion serves to increase the bandwidth of the separating components. In isocratic HPLC, one tries to elute the components before the longitudinal diffusion becomes unmanageable and the peaks are too broad. In gradient elution chromatography, there is an active focusing mechanism (band/peak compression) that dynamically compresses the component bands. The peak width observed in a gradient HPLC run is the product of synchronous diffusion and focusing processes. In a linear solvent gradient, the peak width should be approximately constant throughout the run [12].

The focusing mechanism can be rationalised as follows: imagine a component band broadly diffused on the head of an HPLC column. When a solvent gradient is applied the concentration of organic solvent is always higher at the back of the band than at the front of the band. Hence, the component molecules at the front of the band are more strongly retained by the stationary phase than those at the back. As such, the component molecules at the back of the band are always more

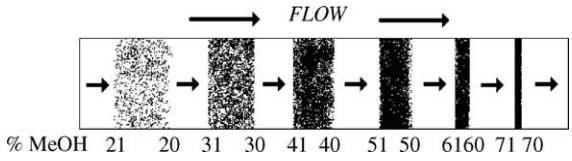

Fig. 1. Schematic of a component band being compressed through a solvent gradient of increasing eluotropic strength. The % methanol figures are arbitrary values to illustrate that a higher methanol concentration always prevails at the back of the component band irrespective of its position in the column.

mobile, any that lag behind are immediately picked up by the stronger eluent and moved forward more quickly to catch up with the rest. Provided that the column is long enough and the gradient is steep enough, peak focusing (or *compression*) progresses up to the point whereby the balance of diffusive and focusing processes determines the final peak width, Fig. 1. Longitudinal diffusion is minimised by using smaller diameter spherical particles, e.g. 3 μm rather than 5 μm silica. The band compression mechanism is maximised by increasing the gradient steepness.

The band compression mechanism may similarly focus organic impurities present in the mobile phase. Impurities that show some retention at low-eluent strength may be focused into peaks as the gradient progresses. This same "trace enrichment" mechanism is exploited in environmental analysis, where pesticides and organic residues are loaded onto a column from a large volume of aqueous sample and then eluted with a focusing solvent gradient [13].

After sufficient focusing, mobile phase ghost peaks appear to be identical to injected analyte peaks, however, where focusing is not complete they may be broader or possess an atypical shape.

Fig. 2 shows how by a focusing mechanism, impurities at levels around 1 ng/mL in the mobile phase may be observed in a blank gradient run. Although this dioctyl phthalate ghost peak is small, it may still interfere with the quantification of low-level organic impurities in a pharmaceutical analysis.

The ideal gradient HPLC mobile phase arriving at the top of the column should contain only the intended solvents and reagents and absolutely nothing else. Gradient HPLC is not

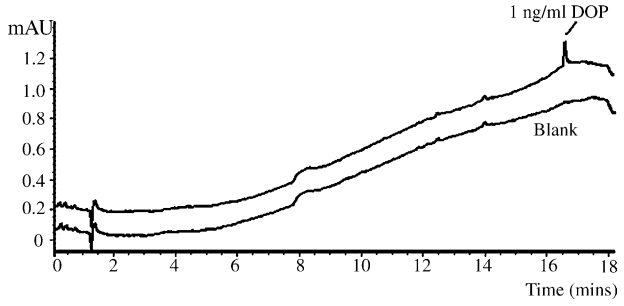

Fig. 2. Chromatogram of eluent spiked with dioctyl phthalate (DOP), Column: Zorbax XDB-C8, 150 mm × 4.6 mm, 5 μm, temp. 40 °C, 1.5 mL/min, λ = 265 nm. Eluent A: water + 1 ng/mL DOP (as bis-2-ethylhexyl phthalate); eluent B: acetonitrile. Timetable (mins, %B): (0, 20), (15, 95), (17, 95), (17.1, 20), (22, 20).

only the analysis of the sample, but also an analysis of the impurities of the mobile phase and solvent pathway of the instrument. To distinguish real analyte peaks from these system (or ghost) peaks, the examination of blank injections is essential.

3.1.1. Water

Water used for gradient HPLC must be as pure and fresh as possible. It should ideally be free from trace organics, inorganics and particulates. Fortunately, for the busy pharmaceutical analyst commercially available equipment can supply the needs of the laboratory such that the water should not be a concern, however, to get good results these purifier systems must be set up and maintained to high standards. Milli-Q and Elga Maxima units are examples of systems that can provide such quality. Essential components of these systems are first a heavy, activated carbon and 5 μm pre-filter (to remove particulates and lower the chlorine content to sub-μg/mL levels), a reverse osmosis unit (to remove the majority of ionic species) and ultraviolet photo-oxidation to kill bacteria and oxidize organic species [11,14]. Ion-exchange and carbon adsorption media are used to further lower the inorganic and organic content and an ultra-microfiltration unit (0.05 μm on Elga Maxima) is employed to ultimately remove bacteria and fine particulates. The system should be disinfected and the consumable parts replaced at regular intervals. Intermittent recycling should also be operating to regularly re-purify water that stands in the system.

There are many other possible approaches to producing pure water, but few are as practical or effective as the commercial systems described above. Several authors have discounted distillation for reasons of cost and efficiency [14–18] and many low-molecular weight organic compounds carry over in the distillation. Homemade attempts at purification by reversed-phase impurity adsorption often fail because the purification media used is more loaded with impurities than the water itself [19,20]. In addition, adsorption systems may require too much maintenance to run long-term and the breakthrough of impurities always ultimately occurs [18]. The most practical proposal to date has been described by Ringo [21] who recommends the use of disposable polystyrene divinylbenzene extraction disks in standard solvent filtration apparatus. These disks absorb hydrophobic UV active organic compounds from water and aqueous buffer solutions. The size and format of these disposable disks means that they can be easily replaced, or regenerated with clean organic solvent.

McCown et al. [18] have reported that continuous degassing with helium (48 h) or prolonged boiling (3 h) significantly reduces the amount of low-boiling impurities, however these would be impractical procedures for many users and neither approach removes high-boiling impurities.

Some in-line HPLC mobile phase purification systems have been shown to work effectively, but these generally necessitate the use of a binary pump system and in-line switching valves to enable back-flushing of the filter media [22,23].

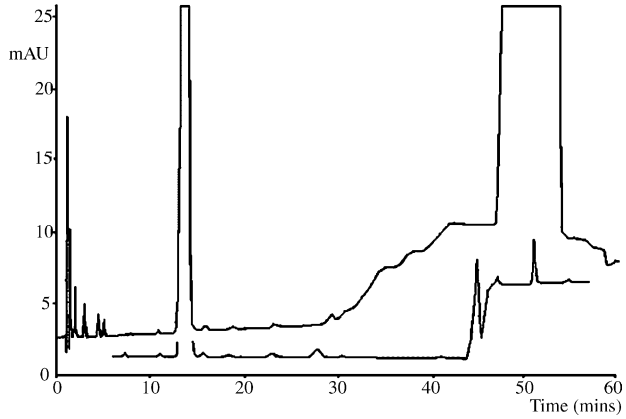

Fig. 3. Comparison of a clean and contaminated sequential isocratic step chromatogram. Column: Genesis C18, 150 mm × 4.6 mm, 4 μm, temp. 25 °C, 1.5 mL/min, λ = 235 nm. Eluent A: 750 methanol, 250 water; eluent B: 900 methanol, 100 water. Timetable (min, %B): (0, 0), (45, 0), (46, 100), (60, 100), (60.1, 0), (70, 0).

Fig. 3 demonstrates the sensitivity of gradient HPLC systems to water quality. The lower trace represents a pharmaceutical related substances method using a "sequential isocratic step gradient" of 75% methanol, stepped up to 90% methanol after 45 min. The upper trace is the same method exhibiting a severe gradient ghost peak that renders the analysis useless. The contaminants begin to break through towards the end of the first isocratic period, and are finally washed out as the eluent strength increases. The reason for this gross system contamination was not fully confirmed, but was strongly suspected to be due to a neglected and bacterially infected water purifier system. The very broad retention range of the impurities is commensurate with the variety of materials produced by bacterial contamination. When bacteria are destroyed their polymeric secretions and lipopolysaccaride cellular fragments remain [24] and this may include polar substances that are not easily removed by adsorption methods [11]. A "biofilm" of bacteria may form inside instruments and tubing if highly aqueous solvents are used continually for long periods. HPLC solvent inlet filters should be replaced regularly or thoroughly cleaned as the high surface area of these makes them a "labyrinthine haven" for bacteria. Periodic flushing of highly aqueous solvent lines with pure methanol or acetonitrile will help to limit microbial growth and further flushing with dichloromethane will fully remove any lipids and grease from the system.

The principal organisms encountered are gram-negative bacteria (e.g. *Pseudomonas* spp.), which can live and grow in pure water [25] and flourish in the presence of feedant materials such as phosphate or acetate. They grow rapidly at 20–35 °C and can be detected in HPLC eluents within just a few of days. Low-temperature storage significantly reduces growth, but it is not normally practical to refrigerate HPLC eluents. Berry [11] has shown that even a pH 9 buffer is not sufficient to prevent growth. Our own microbiologists recommend using mixtures of at least 15% methanol to inhibit bacterial growth and others recommend the use of at least 5% acetonitrile [26]. Berry has mentioned using between 0.04% (w/v) [11] and 0.00004% (w/v) [27] of sodium azide as an anti-bacterial agent in aqueous buffers, although as stock solutions tend to decompose Dolan has described the preparation of 0.004% (w/v) (0.4 g in 10 L of aqueous eluent) for effective use at detection wavelengths down to 210 nm [28]. Some bacteria are resistant to chlorine dioxide, quaternary ammonium compounds and 0.25% acetic acid [25]. Often, they can adapt to harsh conditions, so mobile phase glassware should be regularly cleaned with organic solvent and completely dried, rather than routinely "topped-up" with eluent.

Other anecdotal examples of bacterial ghost peak problems include an in-house case of bacterial growth appearing in an HPLC pump "seal-wash" solution. The organic content of a 50:50 acetonitrile:water seal-wash solution had reduced by evaporation and bacterial growth ensued. Replacement of the "slightly cloudy" seal-wash solution with a fresh mixture completely removed the ghost peaks from the chromatography.

Additionally, and although it may be tempting, one should never use laboratory wash-bottles to make up water volumes for HPLC eluents. This water will not only be saturated with extracts from the wash-bottle plastic, but may also be thriving with microbial life.

3.1.2. Inorganic impurities in water

Modern reversed-phase HPLC columns use ultra-pure (Type B: acid washed, or C: synthetic) silica that is very low in trace metal ions. The performance of the column relies on preserving this low-metal ion content. The water used for gradient HPLC must be fully deionised and purified, not only for the sake of the column, but also because of the effect that the ionic content can have on the gradient baseline. Fig. 4 shows how insufficiently deionised and purified water can actually reverse the slope of a gradient baseline. Non-retained UV absorbing anions (e.g. nitrite, nitrate) are most likely to be responsible for this change in absorbance [29].

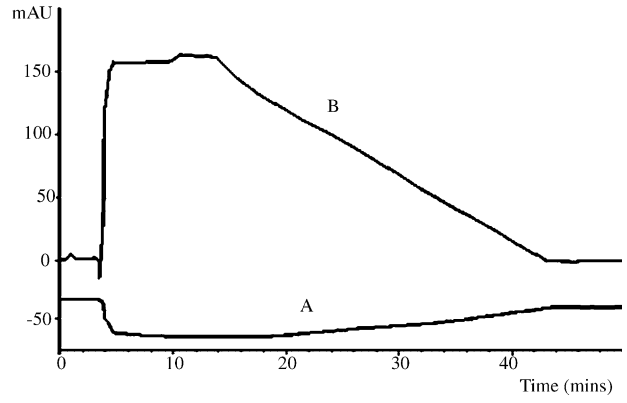

Fig. 4. Gradient baselines obtained using (A) water after purification by an Elga Maxima unit, and (B) water purified by reverse osmosis alone. Column: Hichrom RPB, 250 mm × 4.6 mm, 5 μm, temp. 25 °C, 1 mL/min, λ = 220 nm. Eluent A: water; eluent B: methanol. Timetable (mins, %B): (0, 33), (10, 33), (40, 93), (50, 93) (note, no re-equilibration time.).

3.1.3. Organic solvents

As Dolan has suggested [9] it does seem illogical that impurities in the organic solvent would be concentrated on the reversed-phase HPLC column and then eluted by a trace enrichment and focusing mechanism. However, one has to remember that in the early part of the gradient the solvent mixture has relatively low overall eluotropic strength, so impurities originating from the organic solvent may still be retained and eluted in the same manner. Indeed, it has been shown that some impurities may be strongly retained with 80% organic and eluted as ghost peaks at 90–100% organic [30,31].

Until realising this we had not considered that the organic solvent quality would be a significant issue. In fact, the range of quality between different suppliers and even between product batches can be very significant. Fig. 5 illustrates a problem that we encountered with a supply of gradient grade methanol.

Two separate bottles of a particular methanol batch gave blank gradient chromatograms exhibiting the grossly contaminated upper trace. However, five different batches of the methanol gave clean blanks as demonstrated by the lower trace; all other chromatographic conditions were the same. At the time, the supplier's own quality control tests for the rogue batch actually showed it to be clean, and although the testing procedure was suitable, contamination of the batch occurred sometime after testing, presumably in the bottling process. Since this incident, the supplier has implemented improved manufacturing and QA controls to prevent a reoccurrence. However, this example illustrates the susceptibility of gradient LC to the quality of commercially supplied material. Rogue material batches may be problematic unless the supplier is aware of the problems of trace contamination and has some effective means to prevent unsuitable product units getting to the consumer.

Methanol is typically made by a catalytic reaction of hydrogen and carbon monoxide on huge scales of up to 80,000 gallons per day. With such simple chemistry, commercial methanol tends to have few undesirable impurities present and the purity of HPLC gradient grade methanol is usually better than that of acetonitrile. However, the gradient baseline

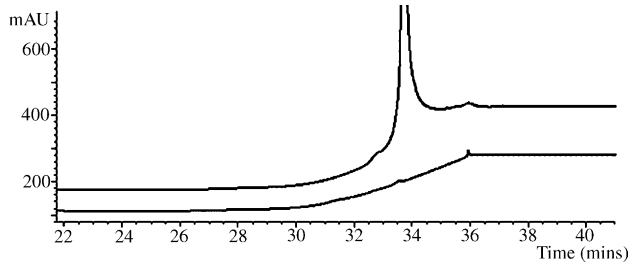

Fig. 5. Comparison of contaminated and clean "Gradient Grade" methanol batches. Column: Zorbax XDB-C8, 150 mm × 4.6 mm, 5 μm, temp. 40 °C, 2 mL/min, λ = 215 nm. Eluent A: water; eluent B: methanol. Timetable (mins, %B): (0, 10), (4, 100), (10, 100), (15, 10), (25, 10), (35, 100), (45, 100), (no re-equilibration time).

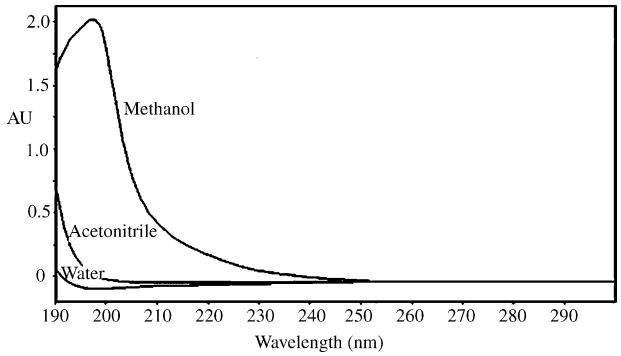

Fig. 6. Absorbance vs. wavelength plots for water, acetonitrile and methanol.

drift observed with methanol is greater than with acetonitrile because of the greater absorbance of methanol in the range of 190–260 nm (Fig. 6).

Thousands of tonnes of acetonitrile are produced every year as a by-product of the acrylonitrile industry [32,33]. The range of impurities in acetonitrile is quite large and the material may pass through a variety of traders and treatment processes before it comes bottled to the hands of the analyst. The range of reported impurities includes acetamide, ethyleneimine, methylamine, ethylamine, benzene, allyl alcohol, acrylonitrile, HCN, acrolein, oxazole, methacryonitrile, butanedinitrile, pentanedinitrile, di-*t*-butylmethylphenol, butylated hydroxytoluene, glutaronitrile, succinonitrile and ammonia [34,35]. Considering the similar chemistry and boiling point some of these impurities it is not surprising that acetonitrile is quite difficult to purify sufficiently well to be suitable for gradient HPLC. Hence, there is a considerable range of qualities available. Acetonitrile does however have the benefit of less gradient drift at low wavelength (Fig. 6) and so is often preferred by chromatographers. Assessment of the purity of acetonitrile by examination of its UV spectrum has been well documented [36], but a blank gradient chromatogram gives a far more accurate and practical assessment of the quality for gradient HPLC [18,37].

Further in-house purification of acetonitrile using alumina columns has been shown to work with varying degrees of success [11,18], some impurities are easily removed and some are not. Hydrogen bonding nitrogenous impurities (e.g. amines and imines) are well retained on alumina columns, but less polar impurities are hardly retained at all. For many impurities in acetonitrile there are not many practical in-house purification options available as there are no sorbents strong enough to retain the impurities in the presence of acetonitrile itself. We have tried C18 and HyperCarb columns without success. Purification is probably best left to specialist manufacturers using distillation and oxidative processes on larger scales [34].

Bristol [31] has described an HPLC procedure to distinguish whether ghost peaks originate from the water or organic solvent of a binary system. To evaluate impurities originating from the aqueous component one must load different volumes of 100% water (or aqueous buffer) onto the column,

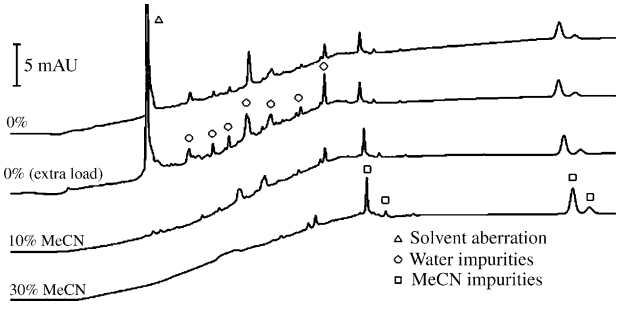

Fig. 7. Determining the origin of eluent ghost peaks caused by impurities in water and acetonitrile. Column: Zorbax XDB-C8, 150 mm × 4.6 mm, 5 μm, temp. 40 °C, 2 mL/min, λ = 215 nm. Eluent A: water; eluent B: acetonitrile. Timetable (mins, %B): (0,{0, 10 or 30}), (10, {0, 10 or 30}), (20, 100), (30, 100), (no re-equilibration time).

then run a gradient and look for an increase in ghost peak height. If one loads 90% water and 10% organic solvent, doubling the equilibration time doubles the loading of impurities from both solvents and gives no useful information. Ghost peaks from the organic solvent can be highlighted by comparing the gradient chromatograms obtained after first equilibrating the system with a 10% organic eluent and then with a 30% organic eluent. Ghost peaks originating from the organic solvent will be larger in the gradient that starts from 30% organic. The increase in ghost peak size is not directly proportional as impurity loading also occurs to some extent during the earlier part of the gradient (see Fig. 7).

The peak labelled "solvent aberration" is derived from a physical mixing effect (or outgassing, see later) caused by the introduction of acetonitrile into a pure water system. This peak has an atypical shape and its area does not change with extra loading of 100% water; the other water impurity peaks double in size. Interesting features in these chromatograms are that, generally speaking, ghost peaks originating from the water tend to appear early in the chromatography and those from the organic solvent tend to be later. It is also notable that a starting mixture of 30% organic has a much smoother baseline than either the 0% or 10% starting conditions, as the mixture is already too strong to efficiently focus the more hydrophilic water impurities.

Tetrahydrofuran, THF, is a very useful solvent modifier for gradient HPLC. Its UV mismatch with water is normally too great for it to be used alone, UV cutoff = 212 nm [38], but it may be used with great selectivity effects at a constant level (1–10%) beneath a gradient of methanol or acetonitrile. As with other solvents, the quality of commercially available HPLC grade THF (unstabilised) is variable and some so-called HPLC grade THF supplies are simply not good enough for gradient HPLC. If BHT (2,6-di-*t*-butyl-4-methylphenol) stabilised THF is used the BHT is easily observed as a well-formed ghost peak typically midway through the gradient.

Fig. 8 shows two offset chromatograms, highlighting the difference between different supplies of (unstabilised) HPLC grade THF. In both cases, a gradient of 10–95% methanol is run with 5% THF held constant in the mobile phase.

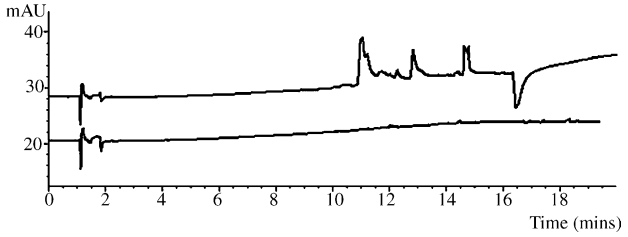

Fig. 8. A comparison of commercially available tetrahydrofuran. The ghost peaks in the upper trace solely originate from the poor quality THF used. Column: Zorbax SB-C18, 150 mm × 4.6 mm, 3.5 μm, temp. 40 °C, 1.5 mL/min, λ = 265 nm. Eluent A: water + 0.1% formic acid; eluent B: methanol + 0.1% formic acid; eluent C, THF + 0.1% formic acid. Timetable (min, %B, %C): (0, 10, 5), (15, 95, 5), (18, 95, 5), (18.1, 10, 5), (23, 10, 5).

In summary, solvents of suitable quality for gradient HPLC are available, but one needs to be vigilant of quality and one may have to examine solvents from several suppliers to find the best.

3.1.4. Reagents and additives for mobile phases

If a gradient chromatogram contains ghost peaks, and it is known that the water and organic solvent are usually of good purity, then any buffer reagent is the next suspect. The more complex the mobile phase, the more chance of impurities and ghost peaks appearing. If an additive is suspected, the eluents should be made up omitting the additives to test their contribution to the problem. Phosphates and acetates are available in a wide range of purity and many are not suitable for gradient HPLC. Filtering the aqueous buffer component with the aforementioned polystyrene divinylbenzene disks may be appropriate here [21]. The quality of commercially available trifluoroacetic acid is also variable and the reagent oxidises with age [39]; older TFA often exhibiting a pale brown colour. Fig. 9 shows a considerable ghost peak at around 13.5 min originating from the TFA used. As with HPLC solvents we endeavour to purchase the best quality available, and this usually involves testing them. We currently find that formic acid tends to be a cleaner reagent for low-pH applications.

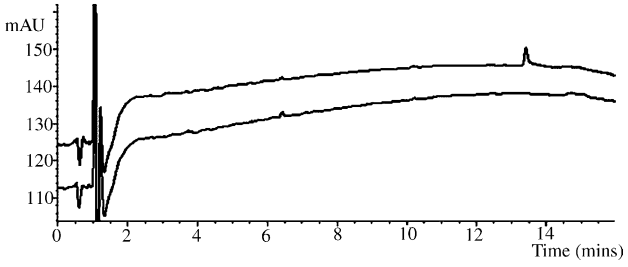

Fig. 9. A comparison of different grades of trifluoroacetic acid. The upper trace uses 0.1% of Aldrich 99% TFA. The lower trace uses Aldrich Spectrophotometric grade TFA. The ghost peak at approximately 13.5 min is a contaminant in the 99% grade TFA. Column: Genesis C18, 100 mm × 4.6 mm, 3 μm, temp. 25 °C, 1.2 mL/min, λ = 215 nm. Eluent A: 1000 water, 1 TFA; eluent B: 900 acetonitrile, 100 water, 1 TFA. Timetable (min, %B): (0, 20), (15, 85), (16, 85), (16.1, 20), (20, 20).

It should be mentioned that some manufacturers are beginning to sell specially prepared buffered solutions for gradient HPLC and they pay close attention to the gradient profile of these solutions.

3.1.5. Phthalates, plastic additives and airborne organics as ghost peaks

Eluents for gradient HPLC should never be kept in plastic containers. The plastic may contain softeners, antioxidants, stabilisers or colours that may leach out of the material and cause ghost peak problems. Gabler et al., observed gradient ghost peak impurities leaching from polyethylene and polypropylene containers into pure water after only one hour [40]. For gradient HPLC the boycott of plasticware should also extend to measuring cylinders, pipettes and wash-bottles.

Nelson and Dolan reported [41] that severe ghost peak contamination occurred when a pH meter probe was inserted into an aqueous buffer solution. The exact origin of the impurities was not defined although as the authors pointed out, it is better to take small aliquots of eluent to measure the pH rather than directly insert a pH meter probe into the mobile phase.

Phthalate esters are semi-volatile liquids used as plasticizers in resins and polymers. In PVC, they may be present in greater than 60% (w/w) [19]. Phthalates have been produced at scales of millions of tonnes per year since the 1940s and are now widely dispersed all over the world and can be detected on almost all surfaces [20] and even within in the Arctic Circle at concentrations ranging from 0.1 to 20 ng/m^3 [42]. They have been detected in and on a wide range of laboratory materials including distilled water, solvents, laboratory air, glassware, vial caps, silica, alumina, filter paper, aluminium foil, Celite, reagents such as sodium sulphate, sodium chloride, and calcium carbonate and ion-exchange materials [19,20,43,44]. Airborne laboratory concentrations of DEHP (bis-(2-ethylhexyl) phthalate) may be up to 35 ng/m^3, depending on the materials used in the building [45].

Using LCMS, we have detected dioctyl phthalate isomers as gradient ghost peaks in HPLC gradient chromatography using both acetonitrile and methanol. In Fig. 10, the diode array TIC response (210–350 nm) for an acetonitrile gradient shows two considerable ghost peaks caused by dioctyl phthalate isomers. These were also observed by direct infusion of the organic solvents (plus 0.1% TFA) into the +ESI mass spectrometer, however the ions were not observed by direct infusion of the HPLC gradient water also containing 0.1% TFA. The structure of the two impurity isomers has not been confirmed and may be caused by any of several dioctyl phthalate or dioctyl terephthalate isomers. The mass attributable to both peaks was 391.2852 Da, compared to a calculated mass of 391.2848 Da. How the phthalates got into these solvents is not clear, however, this chromatography could not be reproduced 6 months later as the contamination of the solvents was probably outside of our control. An innocent change in a manufacturing procedure, the tightness of bottle caps, or the use of a plastic pipe, funnel or container could inadvertently result in solvent contamination. Continuous, long-term production of very high-quality solvents is very difficult and requires careful monitoring of all manufacturing and handling processes.

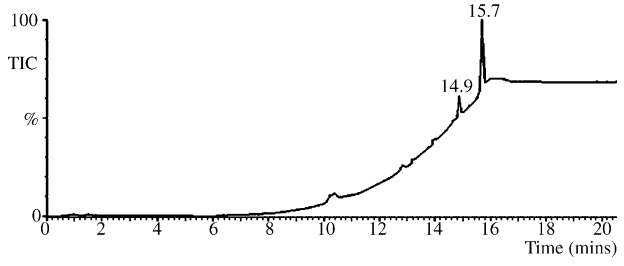

Fig. 10. Gradient chromatogram showing dioctyl phthalate contamination of Gradient HPLC Grade acetonitrile. The peaks at 14.9 min and 15.7 min had identical mass corresponding to different dioctyl phthalate isomers. Column: Zorbax XDB C8, 150 mm × 4.6 mm, 4 μm, 1.5 mL/min, λ = DAD TIC (Total Ion Current). Eluent A: water plus 0.1% TFA; eluent B: acetonitrile plus 0.1% TFA. Timetable (mins, %B): (0, 30), (5, 30), (15, 95), (25, 95), (25.1, 30), (30, 30).

Phthalate levels even on clean glass surfaces tend to increase with time by airborne adsorption [20] and therefore one can assume that absorption into clean solvents exposed to the air would also increase with time. Hence, regardless of any chemical degradation of solvents and reagents, older materials are likely to have absorbed more airborne contaminants than materials that have been freshly purified. We have previously also encountered dibutyl phthalate as an impurity ghost peak, and there are potentially many more plastic and rubber additives and components that may be observed as contaminants [1]. Other organic compounds known to be present in indoor air include surfactants, fire retardants, antioxidants and odorants [46,47]. Similarly, polishes and volatile cleaning products should not be used excessively (preferably not at all) in an HPLC laboratory as we know through our own experience that ghost peaks can be sometimes traced directly to these sources.

3.1.6. Contamination by mobile phase glassware

Untreated glass has an active surface and it can be difficult to remove adsorbed contaminants, particularly detergent residues. Nelson and Dolan reported these difficulties and ultimately recommended 10 rinses of the glassware with tap water and 10 rinses with deionised water to obtain satisfactory results [41]. We have experienced similar difficulties but recommend using very hot tap water, pure water then organic solvent. (The hot water seems to be beneficial in desorbing long chain detergents.)

Cheung and co-workers also found detergent residues interfering with triglyceride assays and reported that they were not removed by multiple water washes or with dilute HCl [48]. One would imagine that in all these cases that the "hardness" of the water may have some influence, hard water washes may be more successful. In his book on Analytical Artifacts, Middleditch discusses 5 or 6 other examples of contaminants being adsorbed to glassware [43].

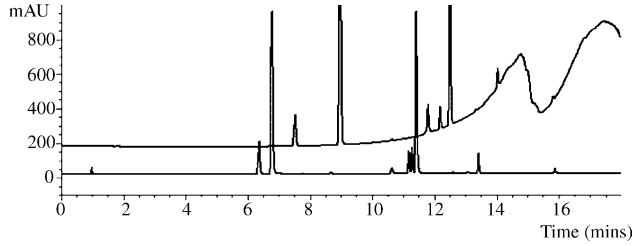

Fig. 11. After standing for several hours the first injection of a sequence using a perfluorinated column (upper trace) and a C18 column (lower trace). Subsequent injections of the perfluorinated column showed less bleed. Columns: 150 mm × 4.6 mm, 3 μm, temp. 40 °C, 1.5 mL/min, λ = 254 nm. Eluent A: water plus 0.1% formic acid; eluent B: methanol plus 0.1% formic acid. Timetable (mins, %B): (0, 10), (15, 95), (18, 95), (18.1, 10), (23, 10).

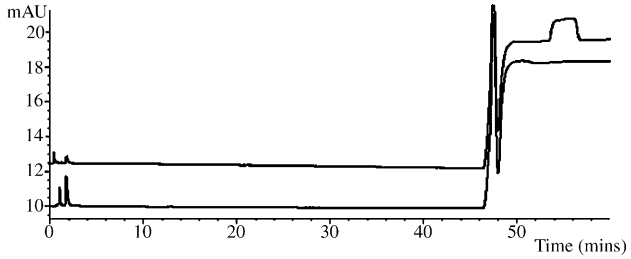

Fig. 12. Different approaches to mixing the same eluent composition, 75% methanol stepping up to 90%. A ghost peak appears at 55 min in the system with a prolonged zero contribution of valve B. Cleaner chromatography is observed if the solvents are mechanically mixed throughout the run. Column: Genesis C18, 150 mm × 4.6 mm, 4 μm, temp. 25 °C, 1.5 mL/min, λ = 235 nm. Upper trace is Eluent A: 750 methanol, 250 water; eluent B: 900 methanol, 100 water. Timetable (min, %B): (0, 0), (45, 0), (46, 100), (60, 100), (60.1, 0), (70, 0). Lower trace is Eluent A: 1000 water; eluent B: 1000 methanol. Timetable (min, %B): (0, 75), (45, 75), (46, 90), (60, 90), (60.1, 75), (70, 75).

Glass remains the most suitable material for the containment of solvents for gradient HPLC, but putting mobile phase glassware through a detergent dishwashing procedure, as many organisations do, tends only to make the glassware dirtier than it would otherwise be. For HPLC eluents we now only rinse the glassware with pure water (if salts are used) then a clean organic solvent. This applies to eluent bottles, measuring cylinders, pipettes and any other glassware used for eluent preparation.

3.2. Other sources of ghost peaks

The first of these is caused by increased "bleed" of the stationary phase under the later and more strongly eluting conditions of the gradient. This is an effect more commonly observed with perfluorinated and phenyl type stationary phases. The example in Fig. 11 is the first injection of a run using a perfluorinated stationary phase compared to the first injection of an identical system using a standard C18 phase. In both cases, the system is allowed to stand for several hours before the run is started. The stationary phase bleed is focused into a broad peak towards the end of the gradient.

Depending on the rate of bleed and the chromatographic method, the problem may be seen only on the first injection and hardly noticeable in subsequent injections, or it may be seen in all the injections of a sequence.

3.2.1. Pumping and mixing problems

In some gradient methods, an isocratic mobile phase is required for a certain period before commencing the gradient. The analyst has options on how to achieve this and the way in which it is done can have unusual effects on the gradient baseline. The gradient method in Fig. 12 requires a mobile phase of 75% methanol for 45 min and then changing to 90% methanol in one minute, and then an isocratic hold for 14 min. Two potential ways of achieving this are:

(1) Prepare eluent A as 75% methanol, and eluent B as 90% methanol and change the solvent delivery from 100% A to 100% B. (Upper trace.)
(2) Prepare eluent A as 100% water, and eluent B as 100% methanol and use the instrument to mix the solvents at 75% and then at 90%. (Lower trace.)

If the mobile phase is pre-mixed as in case (1) an unusual ghost peak at 55 min is observed. This effect was seen on various instruments, usually identical in all the injections in a sequence, but not always so. It sometimes appeared as a square block and sometimes as one or two peaks, and hence it took a very long time to identify the source of this problem. This system had a single pump and a low-pressure mixing valve and it seems that the idleness and delayed start of eluent B was responsible for the observed aberrations. Possible reasons may include valve leakage or failure, dissolved gas problems, or movement of some species into or out of the mobile phase. A high-pressure mixing system also gave a considerable ghost peak at the solvent step, but no delayed aberration as seen with the low-pressure systems. It is clear that in this case it is better to maintain some continuous mixing activity of both solvent lines rather than to leave one resting.

3.2.2. "Air peaks" by injection and mobile phase degassing problems

Ghost peaks that appear virtually identical to real analyte peaks may be caused by the unintentional injection of air into the system.

On many instruments a small plug of air may be present in the needle tip after the sample has been drawn, in a defective injector this may be exacerbated by a siphoning effect that pulls even more air into the injector. Unless a very large volume of air is injected, air injected in this way will be immediately compressed and dissolve into the mobile phase under the pressure of the system. The gas-enriched plug of mobile phase will diffuse longitudinally in the same way as a chemical analyte band as it progresses down the column. Mobile phase saturated with air has a higher absorbance than degassed mobile phase [32] and so a gas-saturated mobile phase peak may be observed that looks very much like a real analyte peak [10].

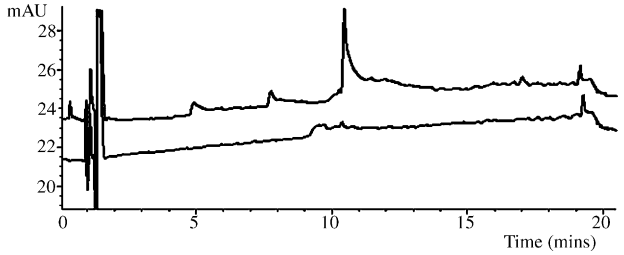

Fig. 13. The upper trace shows a gradient system chromatogram (methanol, 265 nm) with inefficient on-line degassing. After purging the eluents with helium the lower trace was produced. Column: Zorbax XDB C8, 150 mm × 4.6 mm, 5 μm, temp. 25 °C, 1.5 mL/min, λ = 265 nm. Eluent A: 900 water, 100 methanol, 1 formic acid; eluent B: 1000 methanol, 1 formic acid. Timetable (mins, %B): (0, 55), (18, 95), (23, 95), (23.1, 55), (30, 55).

The same problem occurring with aerated sample solvent has also been reported [10]. A difference in the amount of dissolved gas (particularly oxygen) between the sample solvent and the mobile phase produces the same characteristic air peak. Dissolved air peaks may exhibit remarkable retention, with retention times at two or three times t_0 and this can make identification of the problem quite difficult. Confirmation of an air peak problem may be sought by observing changes in the size of the peak when a degassed sample is injected.

Another troublesome problem we encounter sporadically is that of uncharacteristic "shark-fin" shaped peaks with a sharp leading edge and then almost linear decline to the baseline. This type of ghost peak has also been reported in the literature [49]. The size of the peak may be small (only 1–10 mAU) or large (up to 1 AU) depending on the severity of the problem. Fig. 13 shows a gradient of 55–95% methanol (265 nm) over 18 min. The upper trace exhibits several shark-fin shaped peaks caused by a poor efficiency on-line degasser. The lower trace was produced after sparging the solvents with helium. (The rather noisy baseline in this example was due to poor solvent mixing in the delivery system). These peaks are due to transient pressure drops, not impurities. The solubility of air gases is generally greater in pure water and pure organic solvent than in mixtures of the two. Thus, when air-saturated solvents are mixed the mixture tends to have a lower capacity for the dissolved gases and "out-gassing" occurs. In a low-pressure mixing system (where solvent mixing occurs before the pump), this may cause gas bubbles to present themselves inside the pump which results in a pressure drop and a change in absorbance that is immediately registered by the detector. This is usually followed by a steady recovery of pressure, hence the linear tailing. The former scenario is observed several times to different extents in Fig. 13. In severe cases, air bubbles may result in a complete failure of the pump. (This type of manifestation is not normally observed with high-pressure systems as the solvents are not mixed before entering the pumps.) Following the shark-fin peak, the air may then appear as an air peak by the same mechanism as described earlier [32,49]. In addition to degassing the mobile phase we usually add 30–40 cm of PEEK tubing after the detector in the waste line. This adds a few psi of pressure to the flow-cell and reduces the tendency for post-column out-gassing until after the detector.

On-line vacuum degassing for reversed-phase gradient HPLC is usually sufficient, however some manufacturer's systems are better than others, and they can become faulty. Helium sparging tends to be the most effective supporting approach to employ, but one should be aware that "re-gassing" with air begins immediately after helium sparging is discontinued, and that dirty sparging equipment or volatile impurities in the helium line may also contribute to ghost peaks observed in the chromatography.

3.2.3. Injection carryover

If a sample containing highly retained components is injected onto a gradient system that is insufficiently eluting at its end-point, then those components may be eluted during a subsequent injection. To avoid this problem the analyst must endeavour to ensure that the gradient system runs to sufficient solvent strength to elute all of the sample components in a single run. The peak focusing ability of the gradient system may or may not bring a carryover peak to the normal analyte peak width. This problem is easily identified by running the gradient to a higher concentration of organic solvent or by applying a longer isocratic hold at the end of the gradient.

3.2.4. Systematic sample contamination

As with air peaks, sample contamination may cause ghost peaks in both isocratic and gradient HPLC. Strasser and Varadi recently reported two examples of ghost peaks occurring by contamination of the injected sample solution [50]. They found that ghost peaks were observed in the second and later injections from sample vials capped with PTFE-rubber septa, whereas fewer ghost peaks were observed using a more inert PTFE-silicon-PTFE septa. The breaking of the PTFE membrane by the first injection exposed the vial contents to extractable and detectable components of the rubber septa.

In the second example, they found that swab samples used for plant cleaning validation were contaminated with extracts of the latex rubber gloves used by the plant operators. To avoid this, approved glove types were specified in the analytical method.

3.2.5. Ghost peaks by mobile phase component interaction with the stationary phase

Ghost peaks and "vacancy peaks" may be observed by the adsorption or displacement of mobile phase components interacting with the stationary phase. As the complexity of mobile phases and injection solvents increases there is usually an increase in the complexity of system peaks observed at the start of a chromatogram. Such system peaks are normally limited to being very close to the injection front and not at retention factors that most analysts would prefer to have analytes. Reasons for these system perturbations have been discussed in the literature, including the injection front effects observed with systems containing TFA [51] and larger ion-pairing reagents [2,4,52].

3.3. Living with ghost peaks

Sometimes, in spite of one's complete understanding and best efforts to remove gradient HPLC ghost peaks it still might not be possible to completely remove them. If the water, organic solvents and reagents available are just not good enough and cannot be purified further, or the instrumentation cannot be made better there are still some approaches that can minimise the severity of ghost peaks.

(1) If your analysis permits, higher detection wavelengths normally give rise to fewer ghost peaks from solvent mixing, dissolved air peaks, and low-wavelength eluent impurities.
(2) Avoiding low extremes of solvent composition means that hydrophilic mobile phase impurities are not focused as the gradient progresses and the baseline is often smoother.
(3) Clean the glassware and the instrument. As mentioned earlier, mobile phase glassware should only be cleaned with pure water (if using salts) then clean organic solvents. If bacterial contamination of the instrument is suspected then some aggressive cleaning is required, particularly if highly aqueous eluents have been used for a long time. To remove microbial residues, take off the column and flush the system (*but not the detector!*) with 0.5 M sodium hydroxide solution and then with copious amounts of water. The instrument can then be effectively "degreased" by flushing all solvent lines with 100% methanol, followed by 100% dichloromethane, and then again with 100% methanol.
(4) Performing regular blank injections is crucial in reversed-phase gradient HPLC. They provide a regular check on the cleanliness and performance of the system and can help to focus in on the source of troublesome ghost peaks. Ultimately, ghost peaks may have to be selectively omitted from integration in the sample chromatograms.

3.3.1. Electronic baseline subtraction

Ghost peaks can cause considerable problems if they appear at similar retention times to sample component peaks or where they might potentially occur. One way to get around this problem and simultaneously correct the baseline drift is to use electronic baseline subtraction, which is now available on many commercial data processing packages. Berry has previously reported the use of such a technique for gradient HPLC with UV detection at 190 nm [27]. At this wavelength the gradient drift, even with acetonitrile, is very large. Berry observed anomalous dips and peaks in blank subtracted chromatograms due to reproducibility problems between injections under the conditions employed. However, in a gradient system with less severe baseline drift (e.g. when monitored at a higher wavelength) the baseline reproducibility increases markedly. In this case, consistent and robust subtraction of ghost peaks and baseline drift may be possible, however at the moment, methodology using this technique should be validated on a case-by-case basis.

4. Conclusions

Reversed-phase gradient HPLC is an essential and extremely powerful technique in liquid chromatography, however the causes of ghost peaks are generally poorly understood. They are usually caused by the gradient focusing of undesirable, UV active impurities present in the mobile phase. These impurities may still be detected at sub-ng/mL levels and may originate from the water, organic solvent or reagents used to make up the mobile phase. The impurities may be already present in commercial supplies or unintentionally introduced in the laboratory. The concentrations of impurities that appear as ghost peaks may be so low that the commercial specifications of solvents and reagents may not encompass the suitability of the material for gradient HPLC; the quality is therefore not sufficiently controlled and spurious problems may be experienced.

There are also many other reasons why ghost peaks occur, including poor mobile phase degassing, column bleed, injection carryover, injection of air, differences in dissolved air between the sample and mobile phase, systematic sample contamination, and mobile phase component interaction with the column. If ghost peaks cannot be eliminated at their source they may have to be omitted by selective integration of the sample chromatograms, or in specific cases it may be possible to validate an electronic blank baseline correction of the sample data.

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