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PAPER

Perspectives on the future of multi-dimensional platforms†

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Two-dimensional liquid chromatography (2D-LC) formats have emerged to help address separation problems that are too complex for conventional one-dimensional LC. There are a number of obstacles to the proliferation of 2D-LC that are gradually being removed. Reliable commercial instrumentation has become available and data analysis software is being improved. Detector-sensitivity and phase-system compatibility issues can largely be solved by using active-modulation strategies. The remaining challenge, developing good and fast 2D-LC methods within a reasonable time, may be solved with smart algorithms. The technology platform that has been developed for 2D-LC also creates a number of other possibilities. Between the two separation stages, all kinds of physical (*e.g.* dissolution) or chemical (*e.g.* enzymatic or light-induced degradation) processes can be made to take place, allowing a wide variety of experiments to be performed within a single, efficient and automated analysis. All these developments are discussed in this paper and a number of critical issues are identified. A practical example, the characterization of polysorbates by high-resolution comprehensive two-dimensional liquid chromatography in combination with high-resolution mass spectrometry, is described as a culmination of recent developments in 2D-LC and as an illustration of the current state of the art.

1 Introduction to two-dimensional liquid chromatography

Analytical instruments are indispensable for modern society (*e.g.* health, food, environment and materials), for industry and trade, and for research and innovation. Almost every chemical or technological innovation in a food product, material or pharmaceutical formulation is accompanied by analytical confirmation of its efficacy and safety, often followed by additional regulatory requirements. To keep pace with the cumulative needs of society to obtain more objective information and to gain more knowledge, analytical methods are continuously

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improved. From an analytical perspective, the samples subjected to analysis and the questions asked are increasingly complex. To assess the composition and to characterize various properties of highly complex samples, a multitude of analytical techniques are applied. One of these techniques is liquid chromatography (LC).

In LC, analytes are separated as peaks due the differences in partitioning between two phases that move at different velocities. The number of peaks that can theoretically be baseline separated in a chromatographic experiment is referred to as the peak capacity of the method. In cases where the chromatographic experiment is performed under constant (non-programmed) conditions, the following equation applies.

$$n_p = \frac{\sqrt{N}}{4R_s} \ln\left(\frac{1+k_{last}}{1+k_{first}}\right) + 1 \quad (1)$$

Here, k_{first} and k_{last} are the retention factors (or dimensionless retention times), $k = t_R/t_0 - 1$ of the first and last eluting peak, N is the column plate count, and R_s the required resolution. For gradient conditions, the peak capacity is approximately given by

$$n_p \approx \frac{t_G}{4\sigma} \quad (2)$$

where t_G is the duration of the gradient. Literally speaking, n_p is the number of separated peaks that fit in the chromatogram would they be equally distributed. Of course, this is rarely – if ever – the case and in practice the peak capacity must drastically exceed the number of analytes for all of the latter to be separated. According to the statistical overlap theory of Davis and Giddings,[1] the successful separation of 95% of 1000 components would statistically require a peak capacity of 20 000 or more.

The plate numbers and peak capacities offered by LC systems are rather limited when compared with high-resolution techniques, such as gas chromatography (GC) and capillary electrophoresis (CE). Although the performance of LC systems has been improved significantly in recent years thanks to a number of developments, including novel stationary-phase morphologies and ultra-high-pressure technology, the available peak capacities do not suffice to tackle the separation of contemporary complex samples.

While for relatively simple separations data-processing techniques and/or selective detectors may offer refuge, the analysis of truly complex samples with large numbers of analytes becomes extremely challenging. In this case, two-dimensional liquid chromatography (2D-LC) may be fruitful (Fig. 1). In comprehensive 2D-LC (LC × LC), the entire first dimension effluent is subjected to a second dimension separation, split into a sufficiently high number of fractions so as not too lose (too much of) the separation achieved in the first dimension.[2] Very high peak capacities can be achieved and put to use if two very different ("orthogonal") separation methods are coupled. In principle, the peak capacities of the two individual separation systems may be multiplied to obtain the peak capacity, n_c, of the LC × LC method.

$$n_c = n_{p,1} \times n_{p,2} \quad (3)$$

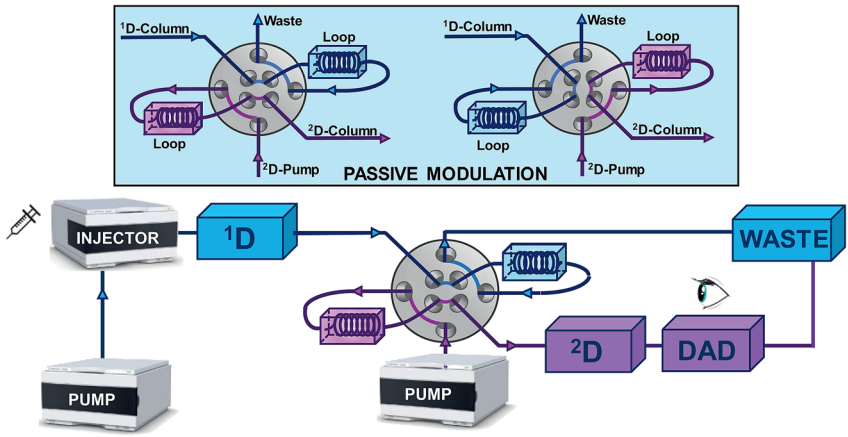

Fig. 1 Schematic overview of a generic comprehensive two-dimensional liquid chromatography (LC × LC) system. The blue colour depicts the first dimension and purple the second dimension. Through the alternating valve-based passive modulation interface, the entire ^{1}D-effluent can be transferred to the ^{2}D separation dimension.

While the essential concepts of 2D-LC were already introduced in the 1970s by Erni and Frei[3] and worked out in 1990 by Jorgenson and Bushey,[4] it was not until the 21st century that developments really started to pick up. There is a clearly identifiable need for comprehensive two-dimensional liquid chromatography (LC × LC) for the characterization of complex polymers that feature multiple independent distributions. This is illustrated by an example of a separation of industrial surfactants in Fig. 2. This created at least one strong driver for developing and improving the technology.[5,6] Because LC × LC offers peak capacities that are about an order of magnitude higher than those attained in conventional one-dimensional LC, the technique may also be advantageous for the separation of very complex mixtures containing 100 analytes or (many) more.[7] For such samples, the combination of LC with mass spectrometry (LC-MS) and LC × LC are both relevant technologies. For the detailed analysis of extremely complex samples, LC × LC-MS may ultimately be the way to go.[8]

Two-dimensional (2D) LC may be applied in the heart-cut mode (LC-LC), in which one or a few fractions are selected for a detailed separation in the second dimension and comprehensive two-dimensional liquid chromatography (LC × LC), where the entire sample is split into many fractions so as to essentially maintain the first dimension separation. All these fractions are subsequently subjected to a second dimension separation, yielding a two-dimensional colour plot that represents the entire sample. While LC-LC and LC × LC techniques were already introduced in the late 1970s, 2D-LC techniques are still not established in most routine analytical laboratories. Massive developments in terms of dedicated, reliable instrumentation for highly efficient (multiple) heart-cut 2D-LC and for (comprehensive two-dimensional) LC × LC have only emerged in the last decade. 2D-LC now appears to be at a stage of development where LC was in the 1980s. The technique is plagued by the perceptions of a reduced detection-sensitivity, limited applicability, lack of robustness, and impracticality due to its complexity.

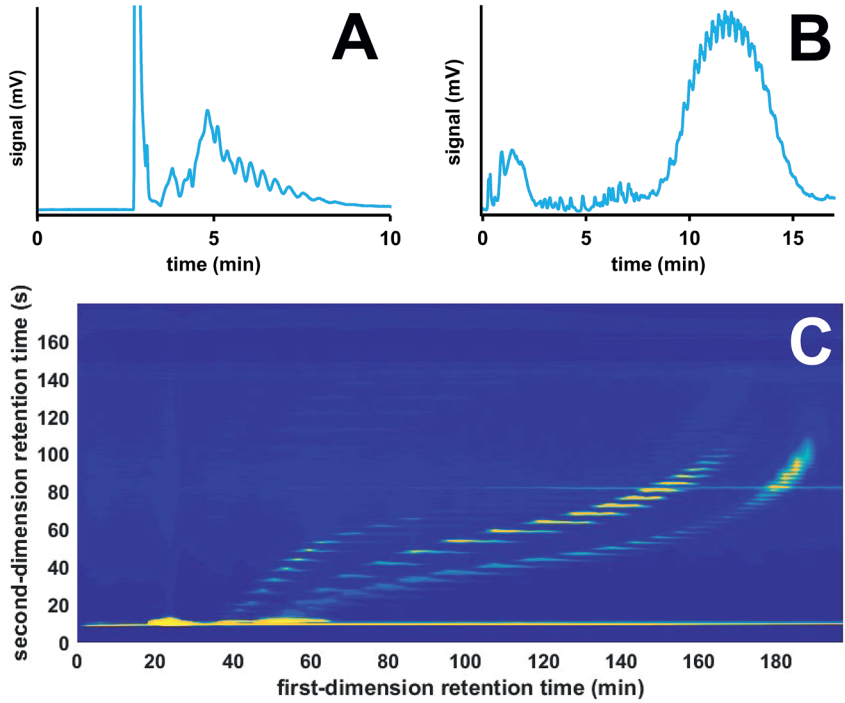

Fig. 2 Separation of a mixture of industrial surfactants using (A) (mixed-mode) ion-exchange chromatography, (B) reversed-phase liquid chromatography, and (C) a comprehensive two-dimensional combination of mixed-mode ion-exchange LC and reversed-phase LC.

We have earlier identified three main challenges as limiting factors for progress in 2D-LC.[9] These are: (i) reduced detection-sensitivity, (ii) limited applicability due to solvent-incompatibility, and (iii) complex and cumbersome method development. Arguably, stationary-phase-assisted modulation (SPAM) has done much to resolve the first issue.[10–12] Significant progress has also been made on the latter two issues.

2 Applicability

In LC × LC, two vastly different (*i.e.* "orthogonal") retention mechanisms are combined. However, many conceivable combinations of retention mechanisms may potentially suffer from incompatibility issues,[7] because the ^{1}D effluent (mobile phase) negatively affects the ^{2}D separation. Active-modulation techniques help negate these deteriorating effects by altering the mobile-phase composition. Moreover, some active-modulation techniques also facilitate peak sharpening to solve the detection-sensitivity problem. The status of these techniques and future prospects are discussed in this section.

2.1 Looking for a breakthrough in overcoming breakthrough

The last decade has seen a steep increase in the development of active-modulation techniques to address detection-sensitivity issues and solvent

incompatibility. Examples include stationary-phase-assisted modulation,[10] vacuum-evaporation modulation,[13] in-column focusing,[14] thermal modulation,[15–17] vacuum-membrane-evaporation and, more recently, although based on earlier developments,[18,19] active-solvent modulation.[20]

While some approaches are still at the pioneering stages, the use of active-modulation techniques is generally seen to be on the rise[2] and this trend is expected to continue in the near future. This will result in improved methods and the exploration of less-common combinations of retention mechanisms.[7]

Because none of the active-modulation techniques has had the time to be fully developed, it is premature to compare their performance or to decide on the strongest options. However, some comments can be made on their current status and on the expected trends and developments heading into the future. The strengths and weaknesses of a number of already implemented active-modulation techniques are listed in Table 1.

The concept of vacuum-evaporation modulation (VEM) and early reports on its functioning are extremely interesting. The technique has specifically been demonstrated for combinations of normal-phase liquid chromatography (NPLC) and reversed-phase liquid chromatography (RPLC).[13] However, it has yet to be demonstrated for LC × LC. Moreover, a thorough investigation into fundamental limitations and possible loss of analytes (discrimination) is very much needed.

Stationary-phase assisted modulation (SPAM) appears to be the most used active-modulation technique[10] and we do not expect this to change rapidly. Incompatible solvents can easily be removed, whilst also improving detection-sensitivity and reducing the analysis time.[7] As a result, we expect more combinations of retention mechanisms to be explored that were previously believed to be incompatible. However, serious attention to the robustness of the traps is

Table 1 Strengths and weaknesses of most used active-modulation techniques

Technique	Strengths	Weaknesses
Stationary-phase-assisted modulation (SPAM)	Eliminates incompatible solvent	Trap robustness
	Improves detection-sensitivity (reduces dilution factors)	Discrimination
	Modulation volume no longer limiting factor	Operation and optimization is sample dependent
		Method development may be challenging
Active-solvent modulation (ASM)	Dilutes incompatible solvent	Modulation volume still a limiting factor
	On-column focusing in the second dimension	Mainly useful with RPLC in second dimension
Vacuum-evaporation modulation (VEM)	Evaporates incompatible solvent	Discrimination: loss of volatile analytes during evaporation not investigated
	Fast operation appears possible (under vacuum conditions)	Some analytes (*e.g.* polymers) may re-dissolve slowly
		Only demonstrated for heart-cut 2D-LC

required. One fundamental limitation of SPAM is the premise that all analytes must be sufficiently retained by the stationary phase in the trapping cartridge.

Active solvent modulation (ASM) is a recently introduced, powerful alternative.[20,21] The concept is founded on a number of papers that have provided a solid basis for further refinement. Given the ease of implementation, we expect ASM to gain in popularity, possibly, to some extent, at the expense of SPAM. ASM requires very high retention factors for the analytes in the weak eluent (*i.e.* the diluted mobile phase). This is easily realized when the ^{2}D separation is RPLC, where the addition of water drastically increases retention factors of almost all analytes, but it is less suitable for most other ^{2}D mechanisms.

All active-modulation techniques have been developed to reduce solvent-incompatibility effects. One serious effect that may jeopardize chromatographic separations is analyte breakthrough. A schematic representation is shown for reversed-phase LC in Fig. 3 where the blue colour depicts the weak (*i.e.* strongly aqueous) solvent and the purple an organic (*i.e.* hydrophobic) solvent. Upon injection, a too strong solvent inhibits adsorption and partitioning of the analytes on or in the stationary phase. Indeed, hydrophobic analytes will essentially move at the same speed as the hydrophobic solvent (*i.e.* the average mobile-phase velocity). As the plug migrates through the column, it is gradually dispersed, and analytes gradually become more retained. However, as long as a plug with a strong, hydrophobic environment pertains, some of the analyte molecules will remain in this zone, to be ultimately eluted with the solvent peak around the dead time of the column (t_0). In cases of gradient-elution experiments, all highly retained analytes that fall behind the solvent plug essentially wait to be caught up by the gradient. For such analytes, breakthrough may affect quantitation (to an extent depending on the volume of the plug), but the chromatogram usually provides a reasonable impression of the contents of the sample. For less-retained analytes, more severe effects on retention and peak shape may be observed.

Breakthrough effects are most serious for large molecules. If the conditions in the plug of strong solvent (and the pore-size distribution of the column) are such that size-exclusion pertains for large (hydrophobic) molecules, they tend to move ahead of the solvent plug, until they meet the weaker solvent. This means that such molecules become focused before the solvent plug. If a plug of strong solvent pertains throughout the column, such molecules elute as a sharp zone, just before the solvent peak. Only molecules that are at the very end of the solvent plug after injection may be able to "escape" from the zone early in the experiment. Once excluded analytes have moved to the front of the zone, there is no escape possible, as long as a zone of strong solvent pertains. In such cases, most of the high-molecular-weight analytes will be found in the breakthrough peak, with only a small fraction as a regular peak later in the chromatogram.

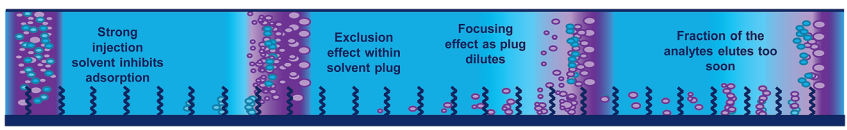

Fig. 3 Schematic representation of the breakthrough phenomena in a reversed-phase column. Here, the blue colour reflects the weak (*i.e.* highly aqueous) eluent, and the purple colour reflects the strong (*i.e.* organic) eluent.

To avoid breakthrough (or any kind of injection-solvent effects) in 1D-LC, the strength of the sample solvent may be lowered, and/or the injection volume may be reduced (Fig. 4). In 2D-LC, the latter can simply be realized by reducing the volume of the modulation loops in any modulator. This may necessitate a lower ^{1}D flow rate, resulting in a longer analysis time and in reduced sensitivity (lower analyte concentrations at the detector). Reducing the strength of the injection solvent is the essence of ASM. By adding a weak ^{2}D eluent to a fraction of modulated ^{1}D strong eluent, the overall solvent-strength is reduced. What is a weak eluent is somewhat dependent on the analytes, but is largely determined by the retention mechanism in the second dimension. To effectively apply these modulation techniques to overcome breakthrough effects, a more fundamental understanding is required.

2.2 Heart-cut 2D-LC to mature more rapidly than comprehensive 2D-LC

We noted in the introduction that the two remaining challenges are solvent-incompatibility and cumbersome and complex method development. The first issue is arguably similar for methods run in heart-cut (2D-LC) and comprehensive (LC × LC) mode. However, method development for heart-cut 2D-LC is much less complex and we believe that heart-cut 2D-LC will more rapidly become established. An investment in dedicated equipment, including a modulation device and a second LC separation, will allow a scientist to couple a familiar 1D-LC separation and select one or a few peaks of interest for further separation.

Heart-cut (2D-LC) has various application domains. It can be used to answer specific questions involving specific analytes, especially in cases where 1D-LC-MS does not suffice. Establishing the purity of an active (pharmaceutical) ingredient is a case in point.[18,22] By separating the product in two orthogonal dimensions, a much better assessment of the purity can be made. Determining enantiomeric ratios in complex mixtures provides another example.[23,24] The complex mixture is first separated, after which the fraction containing the isomers is separated in a second dimension with chiral selectivity. MS cannot usually distinguish between isomers and we believe that chiral LC and chiral supercritical-fluid chromatography (SFC) are, as yet, more powerful than ion-mobility spectrometry (IMS) for such applications.

2.3 Smaller dwell volumes needed

One hardware-related parameter that we believe to be increasingly important is the dwell volume of the chromatographic system. In general, the dwell volume is

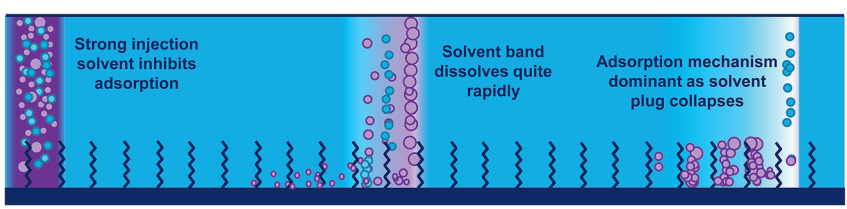

Fig. 4 Schematic illustration of how to prevent breakthrough. The strong sample solvent plug is either sufficiently small or sufficiently weak for the plug to be dispersed to such an extent that the solvent strength falls below a critical level.[56] Analytes will then experience adsorption and will not elute prematurely from the column.

mainly regarded as a nuisance (and a waste of time) for the first dimension separation, due to the low flow rates. Indeed, the flow rates in the ^{1}D separation are generally much lower than those in the second dimension. However, with the introduction of active-modulation techniques, such as SPAM, the restrictions on the ^{1}D flow rate have relaxed. In addition, it should be possible for the dwell volume to be washed away using a high flow rate at the start of the experiment. The volume of liquid may either be flushed through the column prior to injection or diverted away using a valve installed before the column after the injection. The autosampler valve may possibly be used to realize this option.

Ultimately, the ^{2}D dwell volume may be the most important. With the progress of technology (such as SPAM), ^{2}D columns are becoming narrower and ^{2}D flow rates are decreasing. This greatly reduces the solvent volumes needed and the volumes of waste produced. Therefore, we expect this trend to continue. If LC × LC is to be combined with high-resolution MS instruments with interfaces designed to accommodate very low flow rates, such as nano-electrospray ionization (nano-ESI),[10,25] the ^{2}D dwell volume becomes a major bottleneck.

In a well-constructed system, the major contributor to the dwell volume is the mixer, which combines two or more eluent streams into a single homogeneous eluent flow. Efficient mixing is essential, and a certain mixing volume may be fundamentally unavoidable, but mixers should be as small and efficient as possible. Moreover, mixers installed in the eluent delivery system prior to the injector need to withstand very high pressures.

Finally, we would like to emphasize the importance of measuring and knowing the dwell volume for any system. Especially when using computer-aided optimization tools, such as the program for the interpretive optimization of two-dimensional resolution (PIOTR, see Section 3), it is imperative that the dwell volume is accurately known. It can be easily measured by (temporarily) removing the column from the system and measuring the eluent composition in the detector. If necessary, a small amount of a UV-active component, such as acetone, may be added to one of the eluent components for this purpose.

3 (Artificially) intelligent method development

The emergence of robust and reliable instrumentation for 2D-LC and the introduction of active-modulation techniques promise many more successful applications. However, great options, such as variable (*e.g.* shifting) ^{2}D gradients and SPAM, are arguably making the technique even more complex. Having addressed the issues of detection-sensitivity and applicability, the final issue, method development for advanced 2D-LC applications, looms as an enormous mountain on the paths of chromatographers in academia and industry. In this section we will discuss this Everest of all challenges in 2D-LC and the current state of tackling it.

3.1 Introduction to automated method development

The last few decades have seen an increased understanding of chromatographic theory, which resulted in models relating retention to mobile-phase composition for a large number of retention mechanisms, such as reversed-phase LC, normal-phase LC, ion-exchange and hydrophilic-interaction LC (HILIC). These theoretical models can be fitted to experimental retention times of all analytes of interest

from a set of mobile-phase-composition programs. The result will be a retention model for each investigated analyte, from which the retention time can be predicted for any mobile-phase-composition program. By computationally simulating a very large number of methods and assessing the results, the most optimal method can be found.

In a series of papers, we have documented the first steps along what we see as the only way to address the development of complex 2D-LC within a reasonable time (a few days to a week).[26–28] Although significant progress has been made, we can identify a number of further strides that would do much to alleviate the method-development challenge.

3.2 Improving peak-tracking strategies

In computer-aided method development, the peaks do not need to be identified, but we must establish which peaks in the different input chromatograms belong to the same analyte. If this "peak-matching" or "peak-tracking" task is to be performed manually for complex samples, it becomes a painstaking exercise.

We have earlier described our first iteration of a program for automatic peak tracking for use with LC-MS data.[28] During the different stages of testing, our attention became mainly focused on the performance of the program for tracking different isomers present in a sample, which is an indirect indication of the good performance of the system for "easy" analytes (*i.e.* analytes that are substantially different from other ones in the sample). To our surprise, the algorithm also proved to be better than the operators at tracking analytes present at trace-level concentrations. These were frequently missed by the human eye, but unforgivingly spotted by the system. Therefore, we are now more-thoroughly exploring possible routes for testing and using the algorithm as a tool for peak discovery.

Our tracking approach comprises four steps: (i) data preparation, (ii) peak selection (recognition of likely pairs), (iii) comparison, and (iv) evaluation.

The first step encompasses the detection of peaks, followed by removal of noise, background and system peaks. Looking forward, the peak detection and recognition of background signals can still be improved.

In the second step, peaks are classified and pooled as likely or unlikely candidates for matching, depending on the relative retention of peaks in the different input chromatograms. This pre-selection step has mainly been designed and implemented to reduce the time required. To assess all possible peak combinations is quite feasible, but it may be painstakingly slow on regular laptop or desktop computers. All peaks that can be combined in this step do not need to be cross-checked in further steps. However, this step is currently exclusively applicable if the two chromatograms have been recorded using similar chromatographic methods, as is often the case when performing gradient-elution scanning experiments in method-development strategies. One of our aims for the near future is to adapt the algorithm to also allow swift tracking of peaks across chromatograms obtained using very different methods or even across different samples. To accomplish this, the tracking algorithm may be fed with knowledge on the chromatographic mode, the experimental conditions and their presumed (theoretical) effect on the experimental outcome. For example, when comparing an RPLC chromatogram with a HILIC chromatogram (or, more likely when following a systematic optimization strategy, two of each), the system may be aware of the two main parameters influencing retention in the two

modes, which are analyte polarity (inducing peak reversal) and analyte size (promoting a constant elution order). Information on molecular size is expressed in the slope (S) of the RPLC retention curve, which is information that may feasibly be used in a smart peak-tracking algorithm.

The third step involves comparing candidate pairs. It is not dependent on the previous step. In essence, pieces of information are gathered for each peak and these "bits of evidence" are compared for two peaks in different chromatograms. The peak information includes the statistical moments (retention time, area, shape and asymmetry) and the mass spectra. All these bits of information are weighed. Since not all applications use a mass spectrometer, we are actively exploring the use of UV-vis spectra as alternative or additional information on peaks. At a later stage, deep-learning pattern-recognition tools may be investigated to produce additional evidence.

We recognize the need to critically assess the value of each of the pieces of evidence, so as to improve their relative weighing. In this context, it must be immediately noted that some information, such as "the 30 most-abundant peaks in the mass spectrum" and the area (zeroth statistical moment) of the peak, can be significantly influenced by the presence of a nearby, partially co-eluting peak.

The final step involves the evaluation. At this point, alternative pieces of information are consulted with the specific aim to check whether a peak has been sensibly coupled to another peak in another chromatogram. For data involving mass spectrometry, we have tailored this step specifically to a search for possible isomers that are eluting nearby. The algorithm may possibly have matched such peaks incorrectly. In short, in this step the most-abundant mass at the apex is compared between the two peaks. Then, the remainder of the extracted ion-current chromatogram (XIC) is scanned for other signals of the same mass. If other signals representing the same mass exist in the XIC, the elution order in the two chromatograms is compared to verify that the retention order is sensible. If this last test or the apex test fails, the pair is rejected.

While we found this approach to be robust in most cases, one weakness was encountered when an isomer was found to co-elute very closely to another peak of a non-isomer that showed a higher intensity. We are currently adapting the algorithm to improve its robustness in such cases.

In the specific case of LC $\times$ LC, another concern is the speed of the algorithm. As the number of peaks to be evaluated increases, the speed may decrease drastically. In terms of peak-tracking, however, the matching across different retention mechanisms becomes very much easier, because every peak in the 2D chromatogram is physically connected to two retention mechanisms. When matching two or more 2D chromatograms, the additional dimension provides an additional set of statistical moments to include as evidence. It also provides additional information on the retention pattern. The number of options to consider within the search domain of the gradient is actually reduced in comparison with the 1D-LC situation described above. Moreover, co-elution issues are less common and often less problematic. Consequently, it may be expected that the robustness of the two-dimensional version of the algorithm will be better than that of the one-dimensional version.

Ultimately, we may reach a situation in which the algorithm has been refined and optimized to an extent that the processing speed can no longer be reduced. Currently, the algorithm applies a brute-force evaluation strategy. For practical

and fundamental reasons, we want to investigate smart strategies, such as ant-colony and genetic algorithms.

3.3 Polymer separations

Polymers typically feature molecular distributions. There is not a single, unique molecular structure. Almost always, the chain length varies, resulting in a molecular-weight distribution (or molar-mass distribution). In addition, a specific polymer may feature a functionality-type distribution (describing the variation in functional groups or end-groups), a chemical-composition distribution (describing the ratio of monomers in a copolymer molecule), a block-length distribution (in the case of block-copolymers), *etc.*

Therefore, polymer separations involve the separation of envelopes of peaks, which significantly complicates the optimization of polymer separations. Computer-aided optimization, in particular, is complicated, because peak-matching strategies (Section 3.2) have yet to be developed for the envelopes of peaks or "smears" that appear in the two-dimensional chromatograms (or "bananagrams") typically obtained for polymers.[29] One possible direction for such strategies is to use lines to match different smears, including their end points and intermediate points, across different chromatograms (Fig. 5). The PIOTR program may then be adapted to accommodate the optimization of such lines, yielding optimal separations of polymer distributions. Because many, if not most polymeric samples feature more than a single distribution (so called "complex polymers") and because separation dimensions can often be aligned with the limited number of sample dimensions,[30] LC × LC is especially important in the field of polymer separations. Yet, novel developments, such as shifting second dimension gradients and active-modulation techniques, have not yet been embraced. Therefore, there are great opportunities for meaningful improvements, using PIOTR or otherwise.

3.4 Retention mechanism selectors

One frequently raised point of criticism towards the general approach applied in PIOTR is that it requires an *a priori* decision on the selection of retention

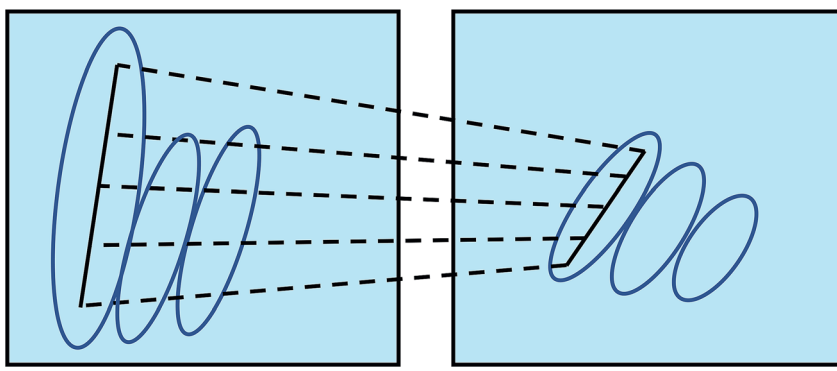

Fig. 5 Polymer distributions usually result in envelopes of peaks in 2D chromatograms. One feasible way to pursue the optimization of polymer separations involves treating them as lines, rather than as individual points (or peaks).

mechanisms. In practice, the selection of suitable and orthogonal retention mechanisms for the sample often involves trial experiments and, therefore, it comprises a significant fraction of the total method-development time for 2D-LC applications.

From a theoretical perspective, the PIOTR approach already contains all the necessary components to combine retention parameters from different 1D-LC retention mechanisms. The user can record 1D-LC data using different retention mechanisms and manually combine these to theoretically assess the efficacy of different combinations through the evaluation of quality descriptions.[7] In its current state, this approach is, however, not effective, because the user must manually match peaks across different mechanisms. This is necessary to use the correct retention parameters for each analyte.

Peak matching is also necessary to perform retention modelling based on experiments carried out with a single retention mechanism (Fig. 6A), but the similarity between the chromatograms thus obtained allows the search window to be narrowed down. For chromatograms obtained using completely different retention mechanisms (Fig. 6B), peak tracking using the approach presented above is still possible, but it is quite slow, because the algorithm cannot focus on a probable retention window, as illustrated in Fig. 6. PIOTR is an efficient program for the optimization of mobile-phase programs. Its usefulness for the selection of retention mechanisms may still be improved.

It is good to realize that peak matching across different dimensions is a non-issue if LC × LC scanning experiments are performed. In that case, peaks in the two dimensions are experimentally matched. Peaks still need to be matched within either mechanism (*i.e.* along the horizontal or vertical axis in the LC × LC chromatogram), but this process may actually be simplified, because the number of candidate peaks may be reduced by information from both axes. The

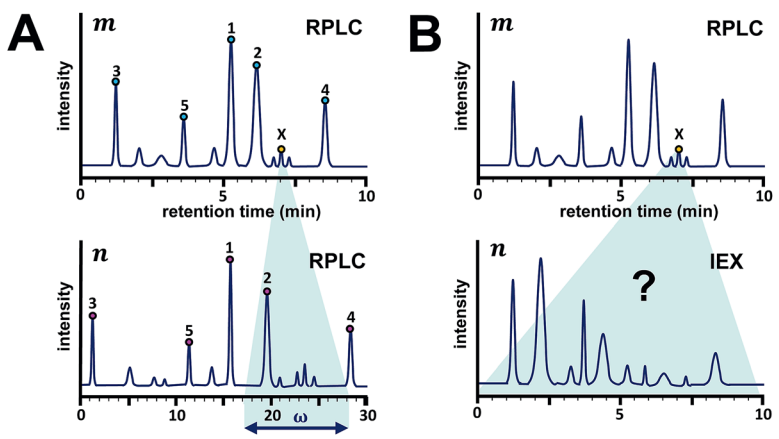

Fig. 6 Tracking peaks across multiple chromatograms is significantly easier when the approximate location of the peak can be predicted. (A) Peak-tracking across chromatograms obtained from similar experiments using identical retention mechanisms, leading to a narrow (initial) search window. (B) Peak tracking of peaks across chromatograms obtained from completely different methods. The search window cannot be narrowed down. The PIOTR approach still works, but more computation time is needed.

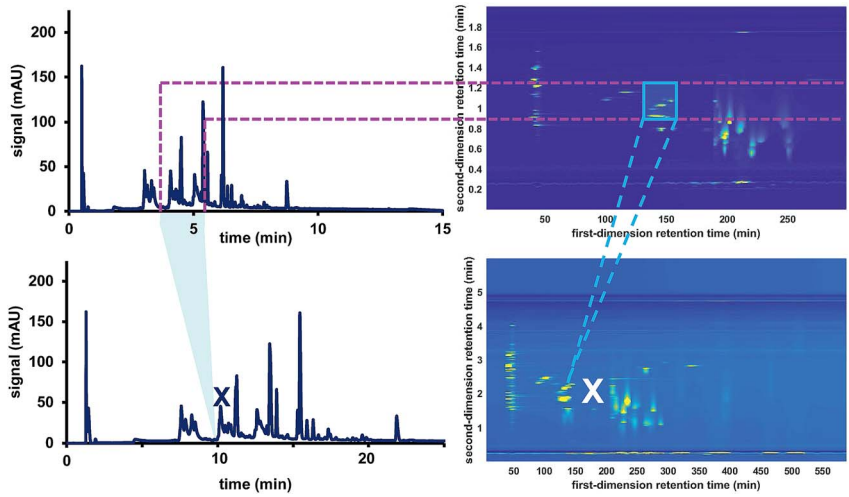

Fig. 7 Even after establishing a narrow search window, plenty of candidate peaks remain during 1D-LC scouting experiments. When using LC × LC for scouting experiments, the search area can be narrowed down in both dimensions, thus significantly reducing the number of candidate peaks.

development of generally applicable LC × LC scouting experiments may be one way to enhance the efficacy of the PIOTR program (Fig. 7).

4 Launching new platforms

The flexible and reliable instrumentation that has emerged for 2D-LC offers a wide range of new possibilities, once it is realized that active-modulation may also include purposeful changes to the sample. Some of the possibilities are discussed in the following sections.

4.1 Physical dissolution

Nanoparticles can feature an array of properties, including their polymer composition,[31] polymer structure,[32] nanoparticle size,[33,34] loading capacity, stability and biodegradability.[31,35,36] These properties can be influenced through several parameters that have been reviewed by Rao and Geckeler.[37] However, methods to simultaneously determine multiple characteristics and their correlation are hard to realize. To establish the relationship between particle-size distribution and molecular-weight distribution for polymeric particles used for coatings, a method was recently developed.[38]

By using very fast size-exclusion-chromatography (SEC) separations[39] and overlapping injections, complete nanoparticle characterizations could be performed in one hour.[38] Fig. 8 displays the configuration used for the first studies on nanoparticles, using a physical-dissolution configuration (PDC), with the inset displaying the situation of the first dimension separation during the initial 12 minutes. Although the setup was successfully used to separate a mixture of polystyrene and polyacrylate particles,[38] there was much room for improvement.

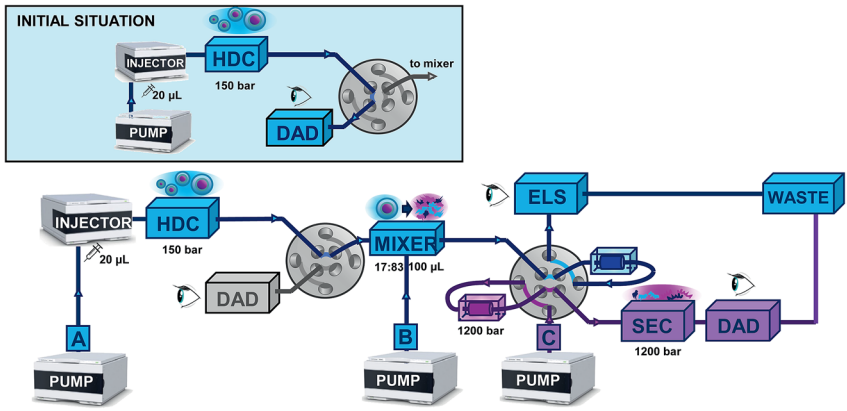

Fig. 8 Example of a physical-dissolution interface, using stationary-phase-assisted modulation traps to reduce dilution effects.

4.1.1 More efficient mixers are needed. One example is the mixing technology. The PDC relies heavily on the efficiency of the employed mixer. At this stage of the study, the band broadening resulting from the very large mixing volumes employed was not critical for proving the concept. However, the development of more efficient mixers is crucial for successful implementation of this technique in industrial practice. This is also true for adapting the PDC to other application fields, such as the analysis of highly complex lignocellulose mixtures or heavy-oil fractions.

Our studies showed a significant influence of the mixing volume.[38] Complete dissolution requires large mixing volumes, which are undesirable from a dispersion perspective. However, only the split-and-recombine approach to mixing was tested. It is recommended to compare other mixing approaches in the search for strategies that require smaller mixing volumes, whilst being more efficient.[40]

4.1.2 Efficient stationary-phase-assisted modulation requires dedicated solutions. A major Achilles heel of the system is its dependence on the stationary-phase-assisted-modulation traps.[38] Sufficient chemical affinity of all analytes for the stationary phase in the trap is important, but this hurdle can usually be overcome. For example, in the setup used for the characterization of poly[(methyl methacrylate)-*co*-(butyl acrylate)-*co*-(methacrylic acid)] nanoparticles displayed in Fig. 9, an additional dilution flow was used to improve retention on the trap columns. More critical than the chemistry of the traps is the significant variation in performance that we experienced. This clearly reduced the robustness of the configuration. Typical problems experienced with the traps were clogging and a significant reduction in retention over the course of a few analyses. It is good to realize that SPAM generally utilizes guard columns, which are essentially developed as disposable consumables. At the same time, these guard columns are used at rather extreme conditions from a column-technology perspective, with exposure to (i) elevated temperatures, (ii) large and frequent pressure pulses, (iii) ultra-high-pressure conditions up to 1200 bar, and (iv) potentially aggressive mobile phases in organic second dimension separations. While our published studies mainly featured guard columns from one manufacturer, we have experienced the

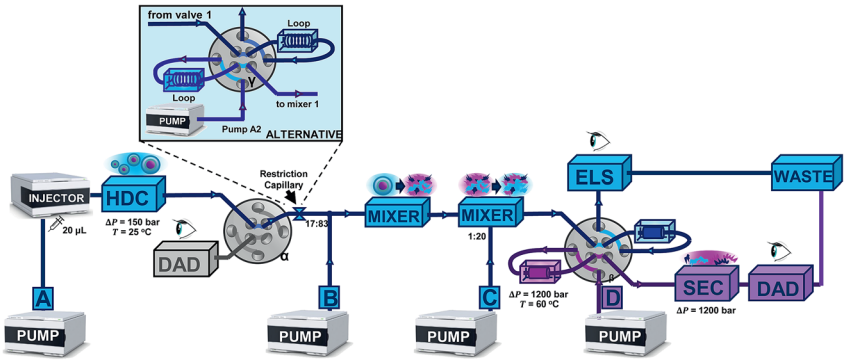

Fig. 9 Setup for the analysis of hydrophilic, charged nanoparticles.

above issues with almost all manufacturers. To reliably use SPAM in routine applications, a dedicated, sustainable solution is needed.

4.2 Enzyme-assisted modulation

4.2.1 Drug-delivery systems. The nanoparticle size, loading capacity, and biodegradability of the polymer are all important for application in controlled-release drug-delivery systems.[31] For example, it is known that small-sized nanoparticles, around 30 nm or below, easily circulate in the human body due to the absence of tissue retention or obstruction,[34] whereas nanoparticles larger than 400 nm may cause hindrances in the vascular system. Capillaries, in particular, may be easily congested by such particles.[34] As explained in a review by Davis *et al.*,[41] particles in the range of 10–100 nm will be optimal for tumour penetration, due to the enhanced permeability and retention (EPR) effect. At the same time, nanoparticles must be sufficiently large to contain a sufficient amount of active ingredient (*e.g.* a 70 nm particle can contain about 2000 small interfering RNA molecules[42]). In essence, it is important that the particle-size distribution (PSD) is well-defined, that the biodegradability is well-characterized, and that the concentration of active ingredient inside the particles is well-known, so that the formulation can be tailored to the application to have the optimal intended effect.

From a chromatographic perspective, correlating particle size with both active-ingredient concentration and polymeric composition comes with another challenge. The particle must be "opened" to release its contents into the eluent. To achieve this inside an organism, nanoparticles created from biodegradable polymers are applied in controlled-release drug-delivery systems.[31,41] Such particles are usually based on poly(lactic-*co*-glycolic acid) (PLGA) and polyethylene glycol (PEG) as PLGA–PEG–PLGA triblocks. PLGA is one of the most successful biodegradable polymers, because its hydrolysis ultimately results in lactic acid and glycolic acid,[31,43] which are both endogenic species. Thus, PLGA is both biodegradable and biocompatible. PLGA also features a number of other attractive properties. It is approved by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) to be used in drug-delivery systems. Moreover, PLGA formulations and methods for production have been well described. Thus, PLGA is the polymer of choice in many current and future drug-delivery systems.

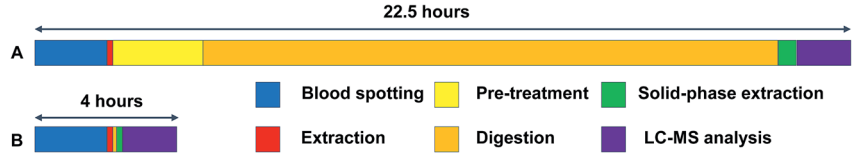

Fig. 10 Comparison of analysis times for dried-blood-spot analysis using (A) in-solution enzymatic degradation, and (B) an immobilized-enzyme reactor (IMER). Using the IMER, the proteins could be digested in 39 s, as compared to 18 h in the case of in-solution degradation. Reproduced with permission from ref. 44.

The biodegradability of the triblock copolymer is largely determined by that of the individual blocks and the overall monomeric composition also plays a role. To characterize the block copolymer, the lengths of the individual blocks must be determined. To this end, physical dissolution does not suffice. However, other forms of reaction modulation may offer a solution.

4.2.2 LC × LC with an immobilized-enzyme reactor (IMER) for nanoparticle analysis. In 2015, Wouters *et al.*[44] investigated the feasibility of enzymes for use in modulators and developed a microfluidic device containing a monolith which was functionalized with immobilized trypsin. Trypsin is an enzyme that is usually used in analytical methods to digest proteins. A trypsin reactor may thus serve as a benchmark for comparison of digestion methods in the field of proteomics. Therefore, initial efforts focused on the degradation of proteins. Wouters *et al.* applied the IMER to the analysis of dried-blood spots and found that it was capable of digesting proteins in 39 s, which compares very favourably with the 18 h required for contemporary in-solution degradation (Fig. 10).[44]

Technically, a similar approach could be applied to polymers. However, the progress in this study was significantly hampered by incompatibility of the enzyme with the typical solvents used to dissolve polymers. One of the first steps in the development of an IMER is the selection of a suitable enzyme. As the interaction of the enzyme with the polymer to be digested can be significantly affected by the morphological state of either, preliminary feasibility studies typically involve testing the enzyme in in-solution-degradation experiments, rather than immobilized in an IMER.

For optimal digestion of a biopolymer, such as PLGA–PEG–PLGA, in solution by an enzyme, (i) the enzyme activity in the solvent must be sufficient, (ii) the polymer must be soluble in the solvent system, and (iii) the enzyme must be able to interact with the polymer (Fig. 11). With some exceptions, most enzymes favour

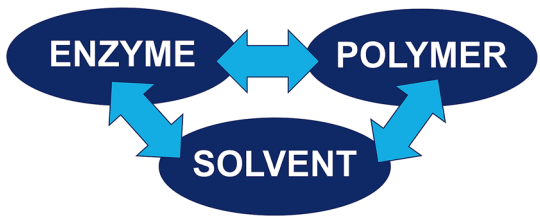

Fig. 11 Compatibility triangle to be taken into consideration during enzyme selection for IMER development.

aqueous systems. However, PLGA–PEG–PLGA polymers are not soluble in such solvent systems. Any efforts to improve the solubility of the polymer in the solvent system, for example by introducing additives, by increasing the organic-modifier content or by adapting the temperature invariably, led to a decrease in the enzyme activity.[45]

In a recent study,[45] nanoparticle dispersions consisting of PLGA–PEG–PLGA polymers were created. Such dispersions are stable in aqueous solvent systems and thus more likely to be compatible with the enzyme. Samples were subjected to various enzymes and were surprisingly well-digested by the enzyme under native conditions in water. The exact mechanism of this process is not yet known. In aqueous solution, PLGA–PEG–PLGA polymers aggregate to form micelles (Fig. 12). The PEG sections of PLGA–PEG–PLGA are relatively hydrophilic and are exposed to the aqueous solvent, whereas the PLGA sections create a hydrophobic core. The investigated enzymes were exclusively able to hydrolyse ester bonds, which exist in PLGA, but not in PEG.[45] This implied that the enzyme somehow managed to penetrate the shell of the particle to break polymer bonds. The energetic interactions between the polar shell and the polar polymers may not be unfavourable. To optimize the IMER-facilitated degradation of PLGA–PEG–PLGA polymers, a better understanding is needed. Ultimately, we want to determine the relationships between the amount of active ingredient, the polymer composition and the particle size in drug-delivery formulations in a single experiment and we are actively investigating possible analytical methods.

Fig. 13A shows one of the setups that was attempted using an IMER in the modulator of an LC × LC system. Using this setup, which is an expansion of the setup shown in Fig. 8, separated particles are subjected to the IMER for degradation. The resulting degradation products may then be trapped in the SPAM cartridges for subsequent analysis by the RPLC second dimension. We are currently testing the feasibility of this system. As a first step, the performance of the IMER, and the viability of trapping and analysing both PLGA–PEG–PLGA-polymer degradation products and the active ingredient using reversed-phase selectivities were investigated. The drug-delivery particles were loaded with

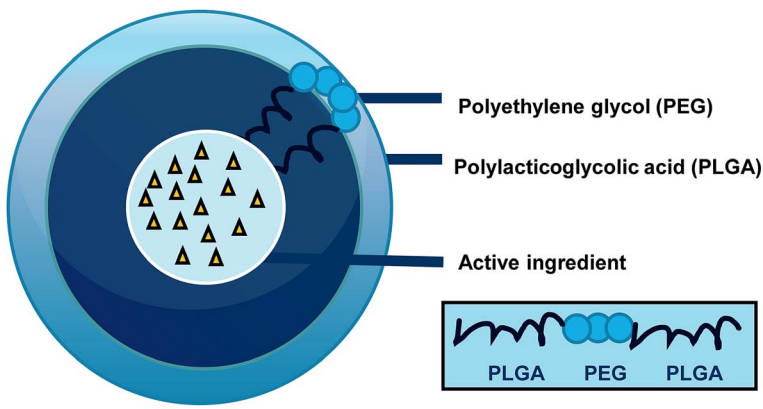

Fig. 12 Illustration of a biodegradable drug-delivery nanoparticle comprised of PLGA–PEG–PLGA polymers and an active ingredient.

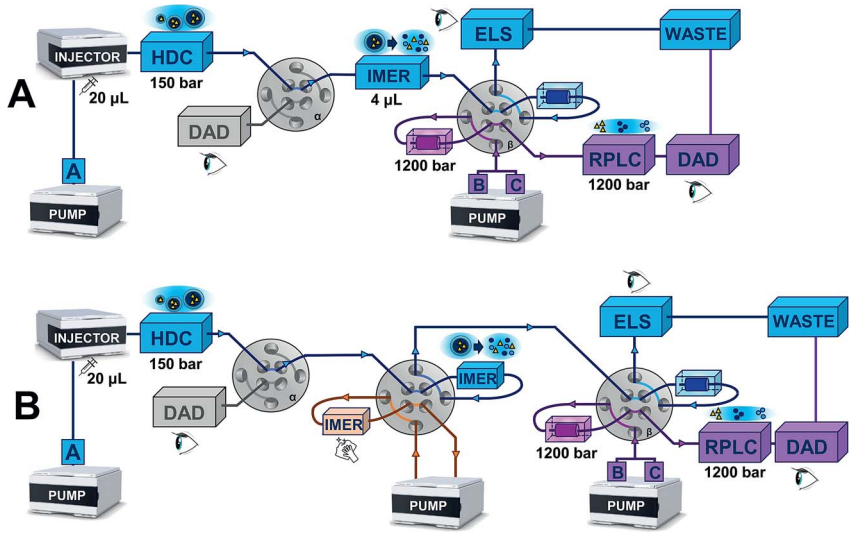

Fig. 13 Two examples of setups explored for on-line reaction modulation of drug-delivery particles using immobilized-enzyme reactors.

a hydrophobic dye. In addition, the HDC column was left out to strictly observe the IMER performance and reduce the number of method variables. An example of a chromatogram resulting from this preliminary study is shown in Fig. 14. As the first dimension HDC column was left out, the first dimension retention time (*x*-axis) merely represents the peak traveling from the autosampler through the IMER to the valve, whereas this normally would show the particle-size distribution of the undigested particles. The second dimension retention time (*y*-axis) reflects the reversed-phase LC separation of the degradation products and the active ingredient, which in this case is the dye. Three peaks can be observed, the latter of which was identified as the dye. One problem at this stage is that most of the degradants are PLGA–PEG–PLGA monomers, which are quite hydrophilic in nature. Such monomers are difficult to separate using RPLC. Therefore, current

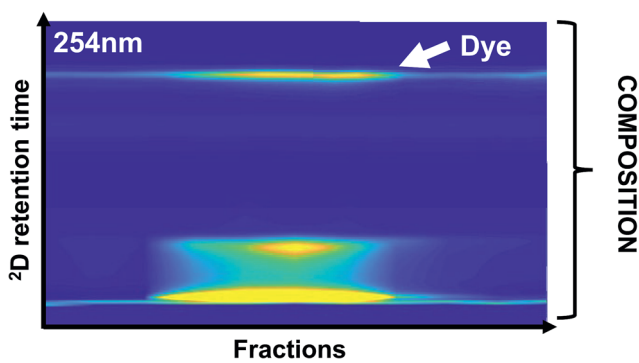

Fig. 14 On-line degradation of dye-loaded PLGA–PEG–PLGA nanoparticles and subsequent RPLC analysis. See text for explanation.

research focuses on finding suitable retention mechanisms, whilst maintaining compatibility with the rest of the separation system.

In addition, there are concerns regarding the stability of the IMER during successive modulations. One solution to regenerate the IMER is the setup shown in Fig. 13B, where the modulation valve can be switched to allow one of the IMERs to be regenerated.

4.3 Dye-degradation mechanisms

Another possibility to manipulate the sample at the modulation stage of an LC × LC experiment is by irradiation. An LC × LC method exists for the separation of dyes.[46] It makes use of a very fast ion-pair chromatography gradient system in the second dimension. The ion-pair agent neutralizes the charged moieties of analytes, greatly increasing their retention in RPLC and allowing their separation in a gradient-elution experiment. In the first dimension, a mixed-mode strong-anion-exchange gradient system was used.

The method has since been expanded to cover virtually all types of natural and synthetic dyes and the selectivity and orthogonality have been optimized using PIOTR.[47] The latter allowed the successful implementation of shifting-gradient assemblies, such that the mobile-phase composition program gradually shifts from (second dimension) run to run. The significant increase in separation power offered by the optimized LC × LC method for separating dyes has led to many more analytes being detected than hitherto possible. A substantial number of these – including analytes present in the extracts obtained from historical cultural-heritage objects – are not identified at this stage.[47] We envisage that the new LC × LC method will make it possible to perform detailed on-line dye-degradation research. A number of off-line strategies exist, but – as explained in Section 4.2 – the implementation of reaction modulation in LC × LC creates exciting new options. For example, a first dimension separation may be used to introduce dyes separately to an exposure cell for light-induced degradation, after which the degradants can be subjected to a second dimension separation for further analysis.

It is anticipated that the matrix, temperature, availability of oxygen, light intensity and duration of the exposure are all important parameters.[48] A well-constructed exposure cell and well-controlled experiments using advanced active-modulation techniques are required for a more thorough, fundamental study into dye-degradation. This should yield qualitative information on degradation pathways and products, and possibly estimates of degradation rate constants.

4.4 Proteoforms

Proteomic studies are typically classified as either top-down or bottom-up proteomics. In the first case, intact proteins are studied, so that information can – in principle – also be obtained on proteoforms (slightly different molecular variations of the same protein). Top-down proteomics has the main advantage that the complete protein sequence can potentially be obtained.

In bottom-up proteomics, the proteins are first digested to peptides. The peptides are then analysed and conclusions are derived about the original protein. This is the most mature and widely used approach for protein

identification and characterization. The disadvantage is that only a fraction of the peptide population of a protein is identified and that, consequently, only a limited section of the protein sequence can be obtained. Bottom-up proteomics becomes increasingly more difficult with increasing complexity of the initial protein mixture.

One opportunity is the use of immobilized-enzyme reactors for the analysis of proteins in both their intact form and through their peptide fragments. The intact proteins can first be separated by either RPLC or hydrophobic-interaction chromatography (HIC), after which the eluting, separated proteins are transported to the IMER. In the IMER, the protein is rapidly digested to the constituent peptides. The effluent of the IMER can be injected into a second separation dimension, where the peptides are separated (preferably using RPLC) and finally introduced into a mass spectrometer.

This approach has major advantages. First of all, by separating the proteins prior to digestion, the resulting peptides can be more easily related to the protein that they derived from. Secondly, the individual proteins are digested more efficiently by the IMER, so that potentially all peptides can be detected, rather than just a fraction. This should facilitate a better coverage of the entire sequence and better protein identification.

An alternative opportunity is illustrated by a recent publication of the LC × LC separation of intact histone proteoforms[25] by Gargano *et al.* Essentially, an intact-protein separation introduces fractions containing resolved proteins into a second dimension separation, where the proteoforms are separated. The authors used HILIC in the second dimension and they found that extremely shallow gradients were required to successfully separate the proteoforms. Such slow gradients are not optimal for second dimension separations. Moreover, the need for such shallow gradients complicates method development. While computer-aided method development for proteoform separations is feasible, the use of spatial devices may eventually also be fruitful.

4.5 Spatial separations

There is actually an egregious waste of time inherent to column-based LC × LC in that all second dimension separations are performed sequentially. If a modest 100 fractions are taken from the ^{1}D effluent and if the cycle time (the analysis time, plus the preparation time in between successive runs, *e.g.* for column re-equilibration) is 2 min, then the analysis time is already more than 3 h. If we were to include a third dimension to perform comprehensive three-dimensional liquid chromatography (LC × LC × LC) and we cut 100 fractions from every ^{2}D run, we would have a mere one second left to perform the ^{3}D separation. The only group to have performed such a feat is that of Jorgenson,[49] who chose a 5 h size-exclusion chromatography (SEC) separation in the first dimension, a 6 min reversed-phase liquid chromatography separation in the second dimension and a 2 s capillary electrophoresis (CE) separation in the third dimension. For a single experiment to be successfully completed, thousands of CE runs had to be performed in a repeatable and reliable fashion.

An alternative is to resort to spatial LC × LC separations, in which analytes are (at least in the first dimension) not eluted from the column, but separated along the length of a separation medium. A conventional form of a spatial LC × LC

separation is 2D thin-layer chromatography. Another example of a spatial 2D separation is 2D-PAGE, where the first dimension separation is based on isoelectric focussing and the second dimension separation on poly(acryl amide) gel electrophoresis. One major advantage of spatial 2D devices is the ability to run all second dimension separations concurrently, almost completely removing the time constraint on the second dimension. Spatial devices have theoretically been shown to be superior in terms of the maximum attainable peak capacity,[50,51] and the implementation of 3D spatial separations is actively pursued by our group.[52]

In spatial 2D-LC, all second dimension separations are normally run with an identical mobile-phase composition program. However, every protein eluting from the intact-protein separation (see Section 4.4) is expected to require a different shallow gradient to obtain a good separation of proteoforms. It is hard to imagine how shifting-gradient assemblies can be implemented in spatial 2D-LC. One alternative may be to "program" the stationary phase. It is conceptually easy to create ^{2}D channels of gradually different lengths, resulting in a version of spatial 2D-LC that is instantly identified as "panflutography" (Fig. 15). Preparing such devices is not more difficult than preparing regular spatial 2D-devices and detection will be only marginally more complicated. Because monolith technology is predominantly used to create stationary phases in spatial separation devices, it may also be possible to "program" the composition of the stationary phase, for example by polymerization at gradually shifting temperatures or by performing grafting reactions with gradually shifting light intensities (in a transparent device).

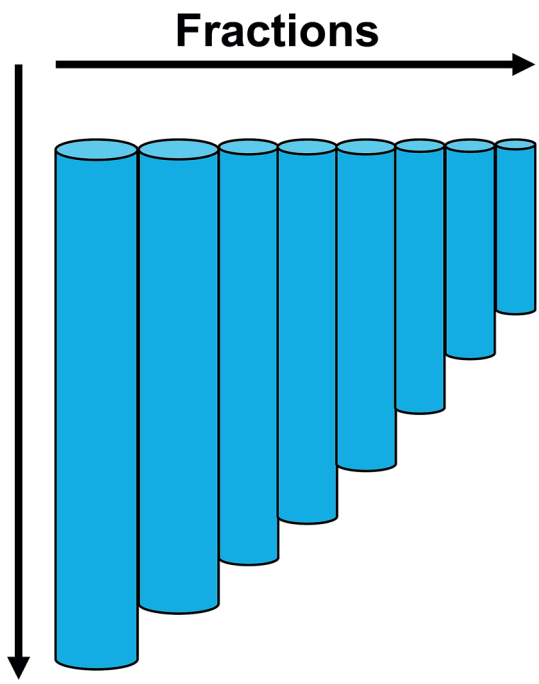

Fig. 15 Crude scheme of a spatial 2D-LC device with varying 2D channel lengths. This type of separation is referred to as "panflutography".

5 Polysorbate analysis

Polysorbates are a special kind of non-ionic surfactants used in a variety of application areas, including stabilizing monoclonal-antibody (mAb) formulations, emulsifiers in the food industry, and solubilizers in cosmetics. They are produced by the ethoxylation of sorbitan and isosorbide (derived from the dehydration of sorbitol) and subsequent esterification of the –OH termini by fatty acids.[53] Ultimately, a complex mixture of various structures is formed, including ethoxylated isosorbide mono-/di-esters, ethoxylated sorbitan mono-/di-/tri-/penta-esters, ethoxylated mono-/di-acids and ethoxylated isosorbide and sorbitan. Depending on the fatty acids used during esterification, the complexity of each group is increased. Furthermore, impurities present in the initiator or fatty acid feeds can increase heterogeneities.

Due to hydrolysis in water-rich environments, the polysorbate can lose its surfactant function, which can be a crucial factor in the stability of mAb formulations and emulsions in food.[54] To characterize which compounds are prone to hydrolysis, complete characterization of the polysorbates is necessary. However, this is a challenging task, since polysorbates are simultaneously heterogeneous in molecular weight and chemical composition.[55]

By combining two separation dimensions, multidimensional characterization of the molecular distributions present in polysorbates was achieved. In the ^{1}D separation, HILIC was employed, resolving the chemical structures based on the degree of ethoxylation. The ^{2}D RPLC dimension resolved the chemical features based on hydrophobicity, *i.e.* ester classes and fatty acid constituents. The HILIC $\times$ RPLC separation was hyphenated with high-resolution mass spectrometry to aid the identification of the separated species.

In Fig. 16, the LC $\times$ LC-HRMS chromatograms are shown of Tween-20 and Tween-80 (Fig. 16A and B, respectively; structures provided in Fig. 17). For information regarding the chemicals used in the experiments, please see ESI Section S-1.† The identified species using the LC $\times$ LC-HRMS data are presented in Table 2. The complexity of the two different samples is clearly presented within the figures, showing group-type clustering of ethoxylated species within the two-dimensional separation space. Overall, ethoxylated isosorbide and sorbitan were detected, as well as ethoxylated mono-/di-acids, isosorbide mono-/di-esters and sorbitan mono-/di-/tri-esters. Within these compound classes, different combinations of fatty acid constituents were detected. These varied between the two Tween samples, which were clearly derived from different fatty acids from different sources. In Tween-20, laurate (12:0), myristate (14:0), palmitate (16:0) and stearate (18:0) species were detected, while myristoleate (14:1), palmitoleate (16:1) and oleate (18:1) species were detected in Tween-80. On average, the isosorbide species have a lower degree of ethoxylation compared to the sorbitan species. This can be explained from the fact that isosorbide has two –OH end-groups available for polymerization, while sorbitan has four –OH end-groups.

Since the two-dimensional separation is highly structured and since the high resolution ensured baseline at many points in the LC $\times$ LC chromatogram, very-low-abundant species were observed in both Tween-20 and Tween-80, which could not be detected in 1D-LC chromatograms. In comparison with isosorbide and sorbitan species, these low-abundant species exhibited an even higher degree

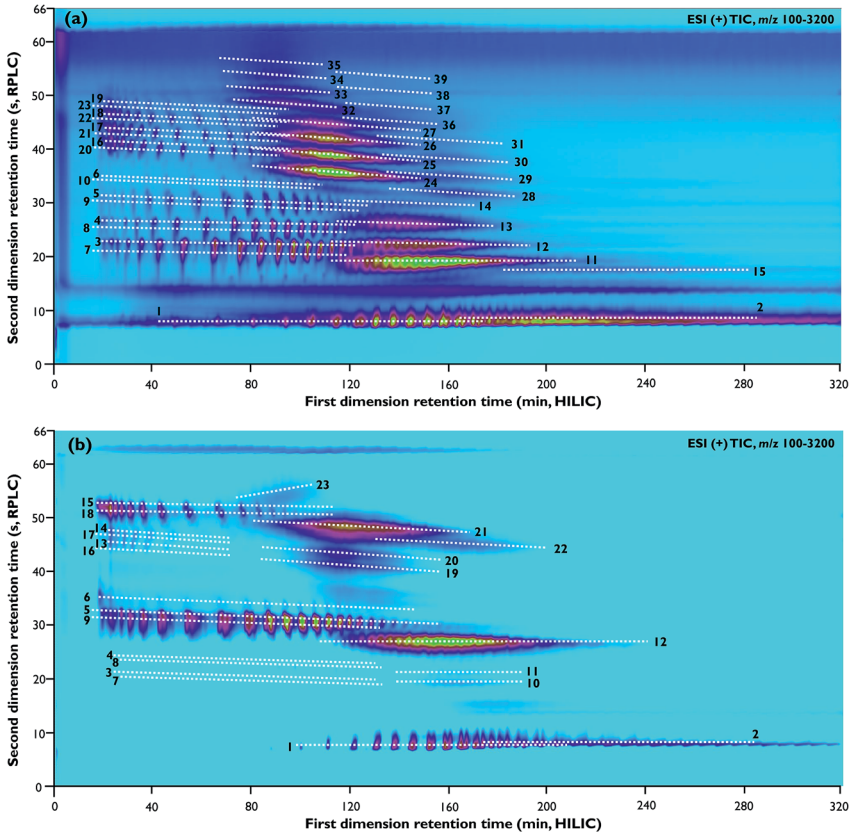

Fig. 16 HILIC × RPLC-HRMS separation of (a) Tween-20 and (b) Tween-80. The degree of ethoxylation is resolved in the ^{1}D HILIC separation, while the ^{2}D resolves the chemical species based on hydrophobicity. Clear group-type separation of isosorbide ethoxylate mono-/di-esters and sorbitan ethoxylate mono-/di-/tri-esters is observed. Within these classes, the species are separated based on the different fatty acid constituents incorporated. Furthermore, sorbitol-initiated species were detected. Identifications are listed in Table 2.

of ethoxylation. Ultimately, the low-abundant species were identified as sorbitol mono-/di-/tri-esters. The sorbitol species may be the result of an impurity left over from the dehydration of the starting compound sorbitol. Such species have not been reported in the literature before, illustrating the power of LC × LC.

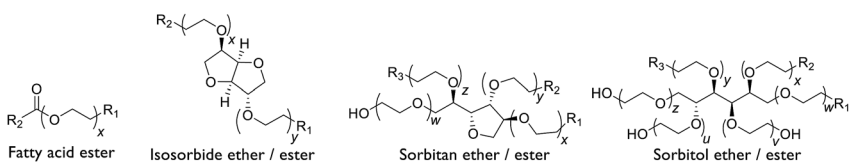

Fig. 17 Generalized structures of the compounds identified in Tween-20 and Tween-80. In Tween-20, R_x = H, laurate (C12:0), myrate (C14:0), palmate (C16:0) and/or stearate (C18:0). In Tween-80, R_x = H, myristoleate (C14:0), palmitoleate (C16:1) and/or oleate (C18:1).

Table 2 Identified chemical species based on the LC × LC-HRMS data as shown in Fig. 16

Tween-20 (polysorbate)			Tween-80 (polysorbate)		
Class	#	Name	Class	#	Name
Polyether polyols	1	Isosorbide ethoxylate	Polyether polyols	1	Isosorbide ethoxylate
	2	Sorbitan ethoxylate		2	Sorbitan ethoxylate
Mono-acid	3	Monolaurate ethoxylate	Mono-acid	3	Monomyristoleate ethoxylate
	4	Monomyristate ethoxylate		4	Monopalmitoleate ethoxylate
	5	Monopalmitate ethoxylate		5	Monooleate ethoxylate
	6	Monostearate ethoxylate		6	Monostearate ethoxylate
Mono-ester	7	Isosorbide ethoxylate monolaurate	Mono-ester	7	Isosorbide ethoxylate monomyristoleate
	8	Isosorbide ethoxylate myristate		8	Isosorbide ethoxylate monopalmitoleate
	9	Isosorbide ethoxylate monopalmitate		9	Isosorbide ethoxylate monooleate
	10	Isosorbide ethoxylate monostearate		10	Sorbitan ethoxylate monomyristoleate
	11	Sorbitan ethoxylate monolaurate		11	Sorbitan ethoxylate monopalmitoleate
	12	Sorbitan ethoxylate myristate		12	Sorbitan ethoxylate monooleate
	13	Sorbitan ethoxylate monopalmitate	Di-acid	13	Monooleate-monomyristoleate ethoxylate
	14	Sorbitan ethoxylate monostearate		14	Monooleate-monopalmitoleate ethoxylate
	15	Sorbitol ethoxylate monolaurate		15	Dioleate ethoxylate
Di-acid	16	Dilaurate ethoxylate	Di-ester	16	Isosorbide ethoxylate monooleate-monomyristoleate
	17	Monolaurate-monomyristate ethoxylate		17	Isosorbide ethoxylate monooleate-monopalmitoleate
	18	Monolaurate-monopalmitate ethoxylate		18	Isosorbide ethoxylate dioleate
	19	Monolaurate-monostearate ethoxylate		19	Sorbitan ethoxylate monooleate-monomyristoleate
Di-ester	20	Isosorbide ethoxylate dilaurate		20	Sorbitan ethoxylate monooleate-monopalmitoleate
	21	Isosorbide ethoxylate monolaurate-monomyristate		21	Sorbitan ethoxylate dioleate
	22	Isosorbide ethoxylate monolaurate-monopalmitate		22	Sorbitol ethoxylate dioleate
	23	Isosorbide ethoxylate monolaurate-monostearate	Tri-ester	23	Sorbitan ethoxylate trioleate
	24	Sorbitan ethoxylate dilaurate			

Table 2 (*Contd.*)

Tween-20 (polysorbate)			Tween-80 (polysorbate)		
Class	#	Name	Class	#	Name
	25	Sorbitan ethoxylate monolaurate-monomyristate			
	26	Sorbitan ethoxylate monolaurate-monopalmitate			
	27	Sorbitan ethoxylate monolaurate-monostearate			
	28	Sorbitol ethoxylate dilaurate			
	29	Sorbitol ethoxylate monolaurate-monomyristate			
	30	Sorbitol ethoxylate monolaurate-monopalmitate			
	31	Sorbitol ethoxylate monolaurate-monostearate			
Tri-ester	32	Sorbitan ethoxylate trilaurate			
	33	Sorbitan ethoxylate dilaurate-monomyristate			
	34	Sorbitan ethoxylate dilaurate-monopalmitate			
	35	Sorbitan ethoxylate dilaurate-monostearate			
	36	Sorbitol ethoxylate trilaurate			
	37	Sorbitol ethoxylate dilaurate-monomyristate			
	38	Sorbitol ethoxylate dilaurate-monopalmitate			
	39	Sorbitol ethoxylate dilaurate-monostearate			

6 Concluding remarks

Liquid chromatography is an immensely successful analytical (and preparative) separation technique. Two-dimensional LC (2D-LC) allows better separation of selected peaks in heart-cut mode or much better separations of complex samples in comprehensive (LC × LC) mode. The application field for 2D-LC is much smaller than that for conventional 1D-LC, but 2D-LC (i) provides a much higher peak capacity (by at least an order of magnitude), (ii) provides additional "orthogonal" selectivity, and (iii) may provide structured, readily interpretable chromatograms. These advantages and the rapidly improving hardware and software provide enough indications that 2D-LC is here to stay – and to grow. The latter will be especially the case if we come to realize that there are a myriad of possibilities to manipulate the sample between the two dimensions. This may be "just" aimed at increasing the sensitivity of detection and the efficiency of the separation (peak capacity) through active-modulation, which also opens possibilities to combine highly different, seemingly incompatible separations. We may also manipulate the sample at the intermediate ("modulation") stage between two LC separations. Nanoparticles may be physically dissolved to allow characterization of particles (*e.g.* the particle-size distribution) and the constituting polymer molecules (*e.g.* molecular-weight distribution) in single on-line experiments. Macromolecules (*e.g.* proteins) may be digested to smaller fragments (*e.g.* peptides) using very fast and efficient immobilized-enzyme reactors and the effects of light on individual sample components (*e.g.* dyes) may be studied by installing an exposure cell in the modulator. Ultimately, LC × LC technology will greatly benefit from a parallelization of all second dimension separations (spatial 2D-LC) and all modulation reactions.

Highly orthogonal and efficient LC × LC-HRMS methods were applied for the characterization of polysorbates, based on the degree of ethoxylation and on hydrophobicity. Highly structured LC × LC chromatograms were obtained and almost all compounds could be identified. Sorbitol ethoxylate mono-/di-/tri esters were detected in very low concentrations.

Conflicts of interest

The authors declare no conflicts of interest.

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