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Recent trends in two-dimensional liquid chromatography

Rick S. van den Hurk [a,b], Matthias Pursch [c], Dwight R. Stoll [d], Bob W.J. Pirok [a,b,d,*]

[a] Analytical Chemistry Group, Van 't Hoff Institute for Molecular Sciences, University of Amsterdam, the Netherlands
[b] Centre for Analytical Sciences Amsterdam (CASA), the Netherlands
[c] Dow, Core R&D Analytical Science, 65201 Wiesbaden, Germany
[d] Department of Chemistry, Gustavus Adolphus College, Saint Peter, Minnesota 56082, United States

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ABSTRACT

Multi-dimensional liquid chromatography (MD-LC) continues to gain in popularity for applications where conventional one-dimensional liquid chromatography is insufficient to solve the analytical problem at hand. In this review we have focused on articles published in the years 2019 to early 2023 and look for trends using our previous review published in 2018 as a baseline. We have also explored usage patterns related to involvement of industrial laboratories in the published research. The two major areas of technical development have been continued work on modulation strategies that help mitigate problems associated with mobile phase mismatch when coupling complementary separation mechanisms, and development of computer-aided method development strategies. Progress in these areas is making 2D-LC easier to use, and it appears that this is translating to a shift toward more involvement by industrial laboratories. Indeed, over 34% of the more than 200 publications on 2D-LC in the last four years have had at least one-industry affiliated author. A recent inter-laboratory comparison study focused on the performance of a sophisticated multi-stage, multi-dimensional separation for therapeutic protein characterization is an exemplary indication of the increasing investment of industrial laboratories to MD-LC, and we expect this trend to continue for the foreseeable future.

1. Introduction

Liquid chromatography (LC) is a ubiquitous separation tool in modern analytical laboratories. Due to its many different separation modes (*e.g.* reversed-phase, size-exclusion, ion-exchange, hydrophilic-interaction, etc.) and because almost all samples can be dissolved in some kind of liquid phase (*e.g.* aqueous or organic), it is a highly versatile technique applied in both academia and industry. For the analysis of highly complex non-volatile samples found in various fields (*e.g.* biopharma, food, environmental, and polymers), traditional LC does not suffice and two-dimensional (2D) LC is an attractive option. Comprehensive 2D-LC (LC × LC) can offer more separation power than conventional one-dimensional (1D) LC, with peak capacities of several thousand [1]. Moreover, when the two selected retention mechanisms are complementary, 2D-LC enables characterization of the sample based on multiple sample dimensions in a single measurement [2]. For details about fundamental principles of 2D-LC, readers are referred to useful guides and reviews published elsewhere [3–6].

Of course, 2D-LC comes with many challenges that have hampered the implementation of the technique in analytical laboratories in industry [5]. Some of these challenges include: (i) insufficient sensitivity; (ii) solvent mismatch problems; and (iii) complexity of instrumentation, software and time-consuming method development. Fortunately, significant progress on each of these fronts has been demonstrated through publications in just the last few years. A point of particular focus in the last decade has been addressing the problem of mismatch between the ^{1}D effluent and the ^{2}D mobile phase that may result in ^{2}D breakthrough; several active-modulation strategies have recently been developed. A popular approach in academic laboratories is stationary-phase-assisted modulation (SPAM) which was introduced by Vonk et al. [7] in 2015. Although this approach is applicable in many 2D-LC configurations, it is not widely implemented in industrial settings due to a lack of robustness. Later, a valve-based approach was introduced by Stoll et al. [8] known as active solvent modulation (ASM). Using a completely different instrumental approach, Schmitz et al. [9] introduced the idea of using an auxiliary pump (a "transfer pump") to push fractions out of sample loops to mix with ^{2}D mobile phase that acts as a diluent. This is similar to the previously introduced At-Column Dilution (ACD) in that it requires an auxiliary pump, but enables continuous variation of the dilution factor [10]. Most recently,

* Corresponding author. Analytical Chemistry Group, Van 't Hoff Institute for Molecular Sciences, University of Amsterdam, the Netherlands.
E-mail address: Bob.Pirok@uva.nl (B.W.J. Pirok).

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Venkatramani et al. [11] have demonstrated the utility of installing an inline mixer between the interface valve and ^{2}D column to mitigate the effects of ^{1}D and ^{2}D mobile phase mismatch. Other active modulation approaches include vacuum-evaporation modulation (VEM) [12] and thermal modulation [13]. For fundamental principles and detailed explanations of these modulation approaches, the reader is referred to the respective article or to reviews published elsewhere [1,6,14–17]. Finally, a recent review describes the opportunities and challenges for hyphenation of LC with supercritical chromatography (SFC) where the modulation interface also plays a crucial role [18].

Despite the remaining challenges related to complexity of implementation and detection sensitivity, industrial laboratories are aware of the potential benefits that multi-dimensional separations can offer and have started investing in research to develop methods tailored to their needs. For example, Goyon et al. [19] recently described the assessment and optimization of a multi-dimensional separation for routine applications, including a wide variety of monoclonal antibody (mAb) products to quantify and monitor post-translational modification (PTM) levels with fast turnaround times. Later Camperi et al. [20] described an interlaboratory study where multi-dimensional separation was investigated for performance and reliability at three different laboratories. And recently, this system was connected to a bioreactor for direct monitoring of post-translational modifications (PTMs) during the cell culture process [21].

In this review, applications of 2D-LC that have been published in 2019 through 2022 are included. A brief overview of modern method development is provided with references to useful reviews. Advances and notable trends compared to the 2D-LC applications from 2016 to 2018 that were reviewed by Pirok et al. [1] are highlighted and discussed. Finally, significant attention is paid to the involvement of industry in recent applications by examining author affiliations of the published work that is covered. The discussion will include some examples of 2D-LC methods that have successfully been implemented in industry.

2. Recent applications

In this section, recently published applications of 2D-LC (from early 2019 through 2022) are discussed; a comprehensive list is shown in Table 1. Applications that used solid-phase extraction as “first-dimension separation” were excluded from the list and considered as (online) sample preparation. The applications are categorized by their overarching field (*e.g.* biopharma, polymers, and food), some of which are discussed in more detail in Section 3. For each application, the following parameters were identified: coupling (online or offline), mode (*e.g.* comprehensive or heart-cut), modulation (*e.g.* passive or active), ^{1}D and ^{2}D separation mechanism, and detection technique. For recent overviews of analyte-specific 2D-LC applications that also include applications published prior to 2019, the reader is referred to the following reviews focused on applications in antibody-based therapeutics [22–24], therapeutic oligonucleotides [25], polyolefins [26], food [27–32], metabolites and lipids [33], amino acids [34], and traditional Chinese medicine (TCM) [35–37].

We have summarized much of the information in Table 1 using a series of graphical overviews (Fig. 1). The layout and colors are the same as those used in similar figures in the extensive review by Pirok et al. [1] (also in Supplementary Material Fig. 1) on 2D-LC applications from early 2016 until late 2018 (with the exception of SFC and Various, they have been modified for visibility); this enables direct comparison of the figures in these two papers, which is helpful for identification of trends. Fig. 1A shows the distribution of different separation mechanisms that are used in the first- and second dimensions of 2D separations. RPLC is still the most commonly used mechanism, represented in 46% and 75% of ^{1}D and ^{2}D separations, respectively. RPLC is used in both dimensions in roughly 33% of all online 2D separations. In these cases, different mobile phases, different stationary phases, or both, are used in the first and second dimensions to provide the complementary selectivities needed to make the 2D separation effective. In cases where non-RPLC mechanisms are used to provide complementary selectivity, these are most commonly used in the first dimension, as this typically simplifies the coupling in online systems [3]. Popular alternative ^{1}D mechanisms include HILIC (18.5%), ion-exchange (10.5%, sum of SAX, SCX, WAX, and WCX altogether denoted as IEX), and SEC (10.0%). RPLC is still dominant in ^{2}D applications as a result of its advantageous behavior in gradient elution (fast re-equilibration). IEX mechanisms may require long equilibration and are used almost exclusively as ^{1}D separations [3]. However, an increase in the use of HILIC in the second dimension of online comprehensive 2D-LC (compared to before 2019) is observed, presumably as a result of technical advances that are discussed in the next section. Overall, the pie charts of Fig. 1A regarding the applied separation mechanisms are highly similar to those from 2016 to 2018 [1].

Fig. 1B summarizes the modulation systems that were used in online 2D-LC applications. By far the most-used modulation approach is passive modulation (67%) which is similar to the fraction observed in applications that appeared in 2016–2018 [1]. SPAM is also still the dominant active-modulation strategy (20%). However, there are some significant changes in the application of the less-explored modulation strategies. Only one publication describing the use of VEM has appeared since the papers that were published before 2019 [263–266]. In the last three years there has been an increase in the use of Transfer Modulation (TM) [9], At-Column Dilution (ACD) [10,195], and related concepts as modulation strategies. The net effect of TM and ACD on mobile phase mismatch is similar to that of ASM but can be applied in different ways. Instead of the valve-based approach to use the ^{2}D eluent to dilute the ^{1}D effluent as in ASM, in TM a separate pump is used to slowly push the ^{1}D effluent fraction into the ^{2}D eluent stream [246]. This results in free adjustment of the online dilution factor but does require an extra pump. Hu et al. [243] used a different approach by omitting sample loops and directly diluting and trapping analytes from the ^{1}D effluent on the ^{2}D column while using two identical ^{2}D columns in place of loops. The method described by Koshel et al. [195] did not use an additional pump but used a two-valve setup for a heart-cut approach. The fraction of published applications involving ASM has not increased substantially. Finally, single applications of both stop-flow and thermal modulation are now included [13,48]. The dominant detection techniques (Fig. 1C) have not changed, and still include UV–Vis absorbance (49.2%) and various types of mass spectrometry (72.1%).

When considering trends related to the application areas, the distribution of non-comprehensive and comprehensive stands out and is depicted in Fig. 2. Pharmaceutical and Biopharma applications are dominated by non-comprehensive techniques over comprehensive ones. This can be explained in part by the necessity to assess impurities around one compound of interest. These applications lend themselves to (multiple) heart-cutting approaches focusing on one or a few analytes. In the fields of energy carriers, food, polymers, and TCM, this trend is reversed and a significant preference towards comprehensive separations exists. In energy carriers and polymers, samples are often distributions of analytes of interest rather than a few specific molecules of interest. In TCM and food, typical fingerprinting of complex mixtures of analytes is required.

3. Technical advances

3.1. Trends in computer aided method development

Historically, multi-dimensional LC method development has been dominated by highly empirical, experience-driven, trial-and-error approaches that have been sufficient in academic environments [267]. However, to make the technique more accessible and attractive to industrial laboratories, systematic and model-driven approaches could streamline method development in multi-dimensional separations in

Table 1
Overview of 2D-LC applications from 2019 until March-2023.

Application	Coupling	Mode	Modulation	^{1}D	^{2}D	Detection	Ref.
Biopharma							
Multi-attribute characterization of adeno-associated viruses	online	HC	SPAM	SAX	RPLC	MS	[38]
Quantification of T-mabs in plasma (adalimumab)	online	HC	Passive	WCX	RPLC	MS	[39]
Antibody-drug conjugates	online	Comp.	Passive	SCX	RPLC	MS, UV–vis	[40]
Hetero-aggregates in Antibody Co-formulations	online	mHC	SPAM	RPLC	RPLC	MS	[41]
Monoclonal antibodies in cell-free culture supernatant	online	HC	Passive	Affinity	RPLC	MS	[42]
Melatonin in cell cultures	online	mHC	Passive	RPLC	RPLC	MS	[43]
Polysorbate hydrolysis in therapeutic proteins	online	HC	Passive	MM	RPLC	CAD	[44]
Enantioselective separation of proteinogenic amino acids	online	mHC	Passive	RPLC	Chiral	MS, UV–vis	[45]
Amisulpridein human plasma	online	HC	SPAM	RPLC	RPLC	UV–vis	[46]
Quantification of phosphorylated tau in cerebrospinal fluid	online	HC	Passive	RPLC	RPLC	MS	[47]
High-purity punicalagin from pomegranate peel wastes	online	mHC	Stop-flow	RPLC	RPLC	UV–vis	[48]
Determination of N-nitrosodiethanolamine	online	HC	Passive	RPLC	RPLC	MS	[49]
Saponins samples from Quillaja Saponaria	offline	Comp.	Manual	RPLC	RPLC	UV–vis	[50]
	offline	Comp.	Manual	HILIC	RPLC	UV–vis	
Analysis of heparinase derived heparin-products	online	mHC	Passive	SAX	SEC	MS, UV–vis	[51]
Interaction between dehydrochlorinated PVC and PEG	online	mHC	Passive	RPLC	HILIC	MS, UV–vis	[52]
Profiling of igg1 producing CHO cells	offline	Comp.	Manual	RPLC	RPLC	MS	[53]
Global Profiling of Lysine Accessibility	offline	Comp.	Manual	RPLC	RPLC	MS	[54]
Charge variant analysis of protein-based biopharmaceuticals	online	Comp.	Passive	SCX	RPLC	MS, UV–vis	[55]
Quality control of synthetic and therapeutic peptides	online	s. Comp.	ASM	RPLC	RPLC	CAD, MS, UV–vis	[56]
Biotherapeutics in serum	online	mHC	SPAM	RPLC	RPLC	MS	[57]
Bridging size and charge variants of a therapeutic monoclonal antibody	online	mHC	Passive	WCX	SEC	UV–vis	[58]
Monoclonal antibodies	online	HC	SPAM	Affinity	SEC	UV–vis	[59]
Monoclonal antibodies	online	HC	Passive	Affinity	SEC	UV–vis	[60]
Antibody characterization (bispecific)	online	HC	Passive	SEC	RPLC	MS, UV–vis	[61]
Therapeutic antibodies	online	Comp.	ASM	RPLC	RPLC	MS	[62]
Multiattribute monitoring of mAbs	online	mHC	ASM	HIC	WCX	MS	[63]
Monitoring of high-mannose glycans of mAbs	online	HC	Passive	Affinity	Affinity	UV–vis	[64]
Purification of biopharmaceutical targets	offline	mHC	Manual	SAX	RPLC	MS, UV–vis	[65]
Complex therapeutic modalities	online	mHC	ACD	SEC	RPLC	UV–vis	[11]
	online	s. Comp.	ACD	IP-RPLC	HILIC	UV–vis	
mAb charge variants	online	mHC	passive	SCX	RPLC	MS, UV–vis	[66]
mAb oxidation	online	mHC	passive	WCX	RPLC	MS	[67]
Energy carriers							
Depolymerized lignin samples	offline	mHC	Manual	SEC	RPLC	UV–vis	[68]
Wastewater of hydrothermal liquefaction of microalgae Chlorella sorokiniana	offline	Comp.	Manual	RPLC	SFC	MS, UV–vis	[69]
Polycyclic aromatic hydrocarbons from vacuum gas oil feedstocks	offline	Comp.	Manual	CPC	SFC	MS, UV–vis	[70]
Separation of carbohydrates from lignocellulosic biomass	online	Comp.	Passive	RPLC	RPLC	MS	[71]
Characterization of sulfur, vanadium and nickel compounds in petroleum products	offline	Comp.	Manual	SEC	RPLC	ICP/MS	[72]
	online	Comp.	Passive	RPLC	SEC	MS	
Lignocellulosic biomass products	offline	Comp.	Manual	CPC	RPLC	MS	[73]
Analyzing lignocellulosic biomass products	offline	Comp.	Manual	SEC	RPLC	MS, UV–vis	[74]
Environmental							
Wastewater treatment in a pharmaceutical plant	online	Comp.	Passive	RPLC	RPLC	MS	[75]
Organic tracers in ice cores	online	HC	Passive	HILIC	RPLC	MS	[76]
Pesticides analysis	online	HC	Passive	HILIC	RPLC	MS	[77]
Extracted pesticides and polar pesticides	online	mHC	SPAM	HILIC	RPLC	MS	[78]
Unknowns in industrial wastewater	offline	mHC	Manual	RPLC	RPLC	MS	[79]
Pesticide multi-screening	online	mHC	Passive	HILIC	RPLC	MS	[80]
Polycyclic Aromatic Hydrocarbons in Water Samples	online	HC	Passive	Affinity	RPLC	FLD	[81]
Chiral pesticides	online	Comp.	Passive	Chiral	RPLC	UV–vis	[82]
Identification of poly- and perfluoroalkyl substances in aqueous film-forming foams	online	Comp.	SPAM	MM	RPLC	MS	[83]
Food							
Phytochemical Characterization of Rhus coriaria L. Extracts	online	Comp.	SPAM	MM	RPLC	MS	[84]
Wine polyphenols	online	Comp.	Passive	TRLC	RPLC	MS	[85]
Determination of vitamin A, vitamin D and vitamin E	online	HC	SPAM	RPLC	RPLC	UV–vis	[86]
Isomeric Flavonoids and Their Glycosides	online	mHC	Passive	RPLC	RPLC	MS	[87]
Determination of mycotoxins, plant growth regulators, tropane alkaloids, and pesticides in cereals	online	HC	SPAM	HILIC	RPLC	MS	[88]
Foenugraeci semen extracts	online	HC	Passive	RPLC	RPLC	MS, UV–vis	[89]
Polyphenolic compounds with biologicalactivity in guabiroba fruits	online	Comp.	Passive	RPLC	RPLC	MS, UV–vis	[90]
Polyphenols from pomegranate juice	online	Comp.	SPAM	HILIC	RPLC	UV–vis	[91]
Classification of olive leaves and pulp extracts	online	Comp.	Passive	RPLC	RPLC	UV–vis	[92]
Mycotoxins in beer	online	mHC	Passive	RPLC	RPLC	MS, UV–vis	[93]
Phenolic analysis in drinks	online	Comp.	ACD	HILIC	RPLC	MS, UV–vis	[94]
	online	Comp.	ACD	RPLC	HILIC	MS, UV–vis	

(*continued on next page*)

Table 1 (*continued*)

Application	Coupling	Mode	Modulation	^{1}D	^{2}D	Detection	Ref.
Impurities from tea leave extracts	offline	mHC	Manual	RPLC	HILIC	UV–vis	[95]
70 regulated and emerging mycotoxins in Pu-erh tea	online	HC	Passive	RPLC	RPLC	MS	[96]
Determination of the phenolic fraction in extra virgin olive oils	online	Comp.	Passive	RPLC	RPLC	MS	[97]
Metabolite Content of Brassica juncea Cultivars	online	Comp.	Passive	RPLC	RPLC	MS, UV–vis	[98]
Polyphenolic fraction of pomegranate samples	online	Comp.	Passive	RPLC	RPLC	MS, UV–vis	[99]
Vitamin D2 and D3 in 9 kinds of edible mushrooms	online	HC	SPAM	RPLC	RPLC	UV–vis	[100]
Astaxanthin-enriched extracts from Haematococcus pluvialis	online	Comp.	Passive	NPLC	RPLC	MS, UV–vis	[101]
Pistacia vera L. Kernel extracts	online	Comp.	Passive	RPLC	RPLC	MS	[102]
Prebiotic oligosaccharides	online	Comp.	SPAM	HILIC	RPLC	UV–vis	[103]
Proteins in eggs	online	Comp.	SPAM	SEC	RPLC	MS	[104]
Gluconic acid in honey samples	online	HC	Passive	SEC	RPLC	MS	[105]
Monoterpene indole alkaloids in biological matrices	online	HC	Passive	SCX	RPLC	MS	[106]
Triazole fungicides in vegetables and fruits	online	HC	Passive	RPLC	Chiral	UV–vis	[107]
Free sterols/stanols and steryl/stanyl esters in walnut	online	HC	Passive	RPLC	GC	MS	[108]
Characterization of bitter peptides in casein hydrolysates	online	Comp.	Passive	SEC	RPLC	UV–vis	[109]
Characterization of proanthocyanidins in cocoa, grape seed and quebracho extracts	online	Comp.	ACD	HILIC	RPLC	MS, UV–vis	[110]
Characterization of phenolic compounds of Algerian date palm leaves and pollen	online	Comp.	Passive	RPLC	RPLC	UV–vis	[111]
Determination of vitamin D2 in mushrooms	online	HC	Passive	RPLC	RPLC	MS	[112]
Studying the energy metabolism of mushrooms (Flammulina filiformis)	offline	mHC	Manual	RPLC	RPLC	MS, UV–vis	[113]
Gelsemium Alkaloids in Honey	online	HC	SPAM	SCX	RPLC	UV–vis	[114]
Separation of minor steviol glycosides	offline	mHC	Manual	RPLC	HILIC	UV–vis	[115]
Phenolic compounds in grape juices and wine	online	Comp.	ACD	RPLC	RPLC	MS, UV–vis	[116]
Polyphenolic content of berry juices	online	Comp.	SPAM	HILIC	RPLC	MS, UV–vis	[117]
Food-derived protein hydrolysates							
Forensics	online	Comp.	Passive	SEC	RPLC	MS, UV–vis	[118]
Characterization of smokeless powders	online	HC	SPAM	SEC	RPLC	UV–vis	[119]
Aging of plastic bonded explosives (PBX)	online	Comp.	Passive	RPLC	SEC	UV–vis	[120]
Lipids							
Profiling of conjugated fatty acid isomers and their lipid oxidation products	online	Comp.	Passive	Chiral	RPLC	MS, UV–vis	[121]
Adult/infant formula analysis	online	Comp.	Passive	RPLC	RPLC	MS, Other	[122]
Profiling of sphingolipids in *Caenorhabditis elegans*	online	mHC	Passive	HILIC	RPLC	MS	[123]
Lipidomics from human plasma	offline	mHC	Manual	HILIC	RPLC	MS	[124]
Separation of lipid species and their oxidation products	online	Comp.	Passive	SEC	NPLC	ELSD	[125]
Lipid profiling in zebrafish embryo	online	Comp.	Passive	RPLC	HILIC	MS	[126]
Cardiolipins and their oxidation products	online	mHC	Passive	HILIC	RPLC	MS	[127]
Gangliosides profiling in swine brain extract	offline	Comp.	Manual	SFC	RPLC	MS	[128]
Characterization of ethanolamine plasmalogens	online	HC	SPAM	NPLC	RPLC	CAD, MS, UV–vis	[129]
Metabolomics							
Metabolome and lipidome simultaneously	online	HC	ACD	HILIC	RPLC	MS	[130]
Metabolomics of human urine	offline	Comp.	Manual	MM	HILIC	MS	[131]
Steroids in human plasma	online	HC	SPAM	HILIC	RPLC	MS	[132]
Sugar phosphates of glycolysis and pentose phosphate pathways	online	mHC	ASM	HILIC	MM	MS, UV–vis	[133]
Cardiolipin oxidation products as a new endpoint for oxidative stress in C. Elegans	online	HC	Passive	HILIC	RPLC	MS	[134]
Determination of N,*N*-dimethyltryptamine (DMT) in rat plasma and brain tissue	online	HC	SPAM	HILIC	RPLC	MS	[135]
Phenolic compounds	online	Comp.	Passive	HILIC	RPLC	UV–vis	[136]
Analysis of 17-Hydroxygeranyllinalool Diterpene Glycosides in Nicotiana tabacum	online	HC	SPAM	RPLC	RPLC	MS	[137]
Miscellaneous							
Differentiation of industrial hemp strains by their cannabinoid and phenolic compounds	online	Comp.	Passive	RPLC	RPLC	MS, UV–vis	[138]
Hydrophobic composition of lignosulfonates	online	Comp.	Passive	HIC	SEC	UV–vis	[139]
Quali-quantitative screening of aqueous phases from pyrolysisbio-oils	online	Comp.	Passive	HILIC	RPLC	MS, UV–vis	[140]
Pendimethalin in tobacco	online	HC	Passive	RPLC	RPLC	MS, UV–vis	[141]
Analyzing human plasma	online	HC	SPAM	RPLC	RPLC	MS	[142]
Aromatic amine oligomer sample	online	Comp.	Passive	RPLC	RPLC	UV–vis	[143]
Qualitative and quantitative analysis of methomyl residue in tobacco	online	HC	Passive	RPLC	RPLC	MS, UV–vis	[144]
Characterizing photodegradation of dyes	online	mHC	Passive	RPLC	RPLC	UV–vis	[145]
Chilling tolerance in grafted cotton seedlings	offline	mHC	Manual	SCX	RPLC	UV–vis	[146]
Peptides							
Identification of naturally processed Zika virus peptides	offline	Comp.	Manual	SCX	RPLC	MS	[147]
Flash frozen and laser microdissected OCT-embedded breast tumor samples	online	HC	Passive	RPLC	RPLC	MS	[148]
Peptide mapping of the complex gastro-intestinal digests	online	Comp.	SPAM	RPLC	RPLC	MS, UV–vis	[149]
	online	Comp.	SPAM	HILIC	RPLC	MS, UV–vis	
Profiling Brain Proteome in Alzheimer's Disease	offline	mHC	Manual	RPLC	RPLC	MS	[150]
Immune Response to *Salmonella enterica* Serovar Typhimurium Infection	offline	Comp.	Manual	RPLC	RPLC	MS	[151]
Sperm proteomic changes	offline	mHC	Manual	HILIC	RPLC	MS	[152]

(*continued on next page*)

Table 1 (*continued*)

Application	Coupling	Mode	Modulation	^{1}D	^{2}D	Detection	Ref.
Complex peptide samples	online	Comp.	Passive	HILIC	RPLC	MS, UV–vis	[153]
Cardiac proteome in a rat model	online	HC	Passive	SCX	RPLC	MS	[154]
Tartary buckwheat (Fagopyrum tataricum) seeds	offline	HC	Manual	RPLC	RPLC	MS	[155]
Microalgae gastro-intestinal digests	online	Comp.	SPAM	HILIC	RPLC	MS	[156]
In-depth proteomic profiling	offline	Comp.	Manual	RPLC	RPLC	MS	[157]
Pharmaceuticals							
Heparinase-products of low molecular weight heparins (lmwhs)	online	mHC	Passive	SAX	SEC	MS	[158]
Automated enantioselective UHPLC screening	online	mHC	Passive	RPLC	Chiral	MS, UV–vis	[159]
High-throughput experimentation with chiral NPLC	online	HC	Passive	RPLC	NPLC	UV–vis	[160]
Pharmaceutical drug oligomers	online	s. Comp.	Passive	SEC	RPLC	MS	[161]
Pharmaceuticals and biomarkers in wastewater-based epidemiology	online	HC	Passive	RPLC	RPLC	MS	[162]
Separation of Warfarin and hydroxylated metabolites	online	mHC	Passive	RPLC	Chiral	UV–vis	[163]
Characterization of impurities and isomers incefpirome sulfate	online	HC	Passive	RPLC	RPLC	MS, UV–vis	[164]
Tropisomers of BMS-986142	online	mHC	Passive	RPLC	RPLC	UV–vis	[165]
Major degradation products in metformin	online	mHC	Passive	SCX	RPLC	MS	[166]
Chiral phosphine ligands and solvent mixtures	online	mHC	Passive	RPLC	Chiral	MS, UV–vis	[167]
Steroids (mix of 17 compounds)	online	Comp.	Passive	TRLC	RPLC	UV–vis	[168]
Racemates and enantiomers (chiral molecules)	online	mHC	Passive	RPLC	Chiral	UV–vis	[169]
Pharmaceutical compounds	online	Comp.	Passive	RPLC	RPLC	UV–vis	[170]
Risperidone and 9-Hydroxyrisperidone in Human Serum	online	HC	SPAM	SCX	RPLC	UV–vis	[171]
Warfarin and hydroxywarfarins	online	mHC	Passive	RPLC	Chiral	MS, UV–vis	[172]
Unknown impurities in azithromycin and erythromycin imino	online	mHC	Passive	RPLC	RPLC	MS	[173]
Pharmaceutical intermediate and its stereoisomers	online	mHC	Passive	RPLC	Chiral	MS, UV–vis	[174]
Impurities in rutin tablets	online	mHC	Passive	RPLC	RPLC	MS	[175]
Impurities in roxithromycin	online	mHC	Passive	RPLC	RPLC	MS	[176]
Twelve posaconazole related stereoisomers	online	mHC	Passive	Chiral	Chiral	UV–vis	[177]
Characterization of Antisense Oligonucleotide Impurities	online	mHC	Passive	SAX	HILIC	MS, UV–vis	[178]
	online	mHC	Passive	IP-RPLC	HILIC	MS, UV–vis	
Screening and analysis of new drug substances	online	Comp.	Passive	RPLC	RPLC	MS, UV–vis	[179]
	online	Comp.	Passive	SEC	RPLC	MS, UV–vis	
	online	Comp.	Passive	SCX	RPLC	MS, UV–vis	
	online	Comp.	Passive	SAX	RPLC	MS, UV–vis	
Various polar and non-polar analytes	online	Comp.	Passive	Chiral	HILIC	CAD, MS, UV–vis	[180]
Determination of valproic acid in human serum	online	mHC	SPAM	RPLC	RPLC	UV–vis	[181]
Pharmaceutical drugs	online	HC	Passive	IP-RPLC	WAX	MS	[182]
Josamycin (degradation products and impurities)	online	mHC	Passive	RPLC	RPLC	MS	[183]
Enoxaparins	online	mHC	Passive	SEC	RPLC	MS	[184]
Metal-based anticancer drugs	online	HC	Passive	SEC	RPLC	ICP-MS	[185]
Impurities in mezlocillin sodium	online	mHC	Passive	RPLC	RPLC	MS	[186]
Characterization of highly polar impurities in calcium gluconate	online	mHC	ACD	IP-RPLC	HILIC	MS, UV–vis	[187]
Microscale isolation of pharmaceuticals	online	mHC	Passive	RPLC	RPLC	CAD, MS, UV–vis	[188]
	online	mHC	Passive	RPLC	Chiral	CAD, MS, UV–vis	
Polymerized impurities in cefmetazole sodium	offline	mHC	Manual	SEC	RPLC	MS, UV–vis	[189]
New bioactive molecules derived from natural products	online	Comp.	Passive	RPLC	Affinity	MS, UV–vis	[190]
Polymerized impuritiesin oxacillin sodium	online	mHC	Passive	SEC	RPLC	MS, UV–vis	[191]
Imatinib and N-desmethylimatinib in Plasma	online	HC	SPAM	SCX	RPLC	UV–vis	[192]
Characterization of synthetic oligonucleotides	online	mHC	Passive	WAX	IP-RPLC	MS	[193]
Synthetic oligonucleotides	online	mHC	Passive	MM	RPLC	MS, UV–vis	[194]
Impurity analysis of dye-conjugated oligonucleotides	online	HC	ACD	RPLC	RPLC	MS, UV–vis	[195]
Polymers							
Propoxylate mixture	online	Comp.	Passive	NPLC	RPLC	ELSD	[196]
Propoxylates with varying hydroxyl end groups	online	Comp.	Passive	NPLC	SEC	ELSD	[197]
Polymeric impurity in block copolymers	online	Comp.	ASM	SEC	RPLC	ELSD, MS	[198]
Thermal modulation of polymers using cold trapping	online	Comp.	Thermal	RPLC	SEC	UV–vis, RID	[13]
Chain walking polyethylene (branching conformation and molar mass)	online	Comp.	Passive	TGIC	SEC	ELSD	[199]
	online	Comp.	Passive	RPLC	SEC	ELSD	
Hybrid water-soluble biopolymers	online	Comp.	Passive	SEC	RPLC	ELSD	[200]
Polyolefin plastomers/elastomers (ethylene/1-octene copolymers)	offline	HC	Manual	NPLC	SEC	ELSD	[201]
High molecular weight multi-arm functionalized peg–maleimide	online	HC	Passive	SEC	RPLC	CAD, MS	[202]
	online	HC	Passive	RPLC	SEC	CAD, MS	
Linear low-density polyethylene resins	online	Comp.	Passive	NA-RPLC	SEC	ELSD	[203]
Charged acrylic polymeric particles	online	Comp.	SPAM	SEC	SEC	UV–vis	[204]
Acrylate-modified hyaluronic acid	online	Comp.	Passive	RPLC	SEC	ELSD	[205]
Synthetic polymers (vinyl acetate/acrylic acid copolymers and vinyl acetate/itaconic acid/acrylic acid terpolymers)	online	Comp.	ASM	SEC	RPLC	ELSD	[206]

(*continued on next page*)

Table 1 (*continued*)

Application	Coupling	Mode	Modulation	^{1}D	^{2}D	Detection	Ref.
Characterizing polymer microstructure for acid-functional polymers	online	Comp.	Passive	SAX	SEC	ELSD	[207]
Advanced polymeric materials	online	Comp.	ASM	SEC	RPLC	ELSD	[208]
Proteins							
Protein expression in mouse brain	offline	mHC	Manual	RPLC	RPLC	MS	[209]
Proteomes of truffles	offline	mHC	Manual	RPLC	RPLC	MS	[210]
Proteomics of human frontal and temporal cotrex	offline	HC	Manual	RPLC	RPLC	MS	[211]
Proteins in human serum	online	mHC	Passive	RPLC	RPLC	MS	[212]
Metaproteomics of Environmental and Microbiome Samples	online	Comp.	SPAM	RPLC	RPLC	MS	[213]
High-throughput identification of protein complexes	online	mHC	SPAM	WCX	RPLC	MS	[214]
Enhancement for bottom-up-proteomics	online	Comp.	SPAM	HILIC	RPLC	MS	[215]
Comparison of modulation strategies	online	Comp.	Passive	HILIC	RPLC	MS, UV–vis	[216]
Purification of natural neutral N-glycans	offline	mHC	Manual	HILIC	RPLC	MS, UV–vis	[217]
Mapping Influenza-Induced Posttranslational Modifications on Histones	online	mHC	Passive	RPLC	MM	MS	[218]
Protein interaction with immunoglobulin G in hela cells	offline	mHC	Manual	SAX	RPLC	MS	[219]
Separation of intact proteins	online	Comp.	Passive	RPLC	RPLC	MS, UV–vis	[220]
Hydrophilic proteins from soy flour samples	online	mHC	SPAM	SEC	RPLC	UV–vis	[221]
Traditional Chinese medicine							
Metabolites characterization of two Astragalus species	online	Comp.	Passive	RPLC	RPLC	MS, UV–vis	[222]
Phenolic acids, saponins, sulfur derivatives, and alkaloids in Lilium	offline	mHC	Manual	HILIC	RPLC	MS	[223]
Chemical profiling of Qingfei Paidu Decoction	offline	Comp.	Manual	HILIC	RPLC	MS	[224]
Multicomponents from Compound Danshen Dripping Pill	offline	mHC	Manual	HILIC	RPLC	MS	[225]
Ginsenoside Contents in Ginseng	online	mHC	Passive	RPLC	RPLC	UV–vis	[226]
Profiling of Sanguisorba officinalis	offline	Comp.	Manual	MM	RPLC	MS	[227]
Structural characterization of dipeptides of Cordyceps sinensis	offline	Comp.	Manual	HILIC	RPLC	MS	[228]
Antibacterial constituents in medication	online	Comp.	Passive	RPLC	RPLC	UV–vis	[229]
Chemical components of Panax notoginseng leaves	online	HC	SPAM	RPLC	HILIC	MS	[230]
Polar compounds from Saussurea obvallata	offline	mHC	Manual	RPLC	HILIC	UV–vis	[231]
Multicomponent characterization of Gastrodia Rhizoma	offline	Comp.	Manual	HILIC	RPLC	MS	[232]
Chemical distinction of Chrysanthemum species	online	Comp.	Passive	RPLC	RPLC	MS, UV–vis	[233]
Characterization and quantification of the non-polysaccharides in Sijunzi decoction	offline	mHC	Manual	RPLC	RPLC	MS	[234]
Characterization of steroid alkaloids in Fritillariae Pallidiflorae Bulbus	offline	Comp.	Manual	HILIC	RPLC	MS	[235]
Multi-herb Chinese medicine formula	online	Comp.	Passive	RPLC	RPLC	MS	[236]
Characterization of Atractylodis Macrocephalae Rhizoma	offline	mHC	Manual	HILIC	RPLC	MS, UV–vis	[237]
White ginseng and red ginseng	offline	Comp.	Manual	HILIC	RPLC	MS	[238]
Dendrobium species included D. Officinale, D. Nobile and D. Chrysotoxum.	online	Comp.	Passive	RPLC	RPLC	MS, UV–vis	[239]
Toad venom, dammar resin, and propolis	online	Comp.	VEM	NPLC	NPLC	UV–vis	[240]
All-trans-,9-cis-,and13-cis-astaxanthin in raw extracts from Phaffia rhodozyma	online	HC	Passive	RPLC	RPLC	UV–vis	[241]
Ginsenoside Biotransformation in Panax notoginseng Inflorescences and Leaves	online	Comp.	SPAM	HILIC	RPLC	MS	[242]
Alkaloids in Macleayacordata (willd.) R. Br	online	Comp.	ACD	RPLC	RPLC	MS	[243]
Chemical characterization of Lonicerae Japonicae Flos	offline	Comp.	Manual	HILIC	RPLC	MS	[244]
Accurate determination and quality evaluation of Chinese propolis	offline	HC	Manual	RPLC	RPLC	MS	[245]
Metabolite analysis of Buddleja davidii	online	Comp.	ACD	RPLC	HILIC	MS	[246]
Panax ginseng root extract in saponin	online	Comp.	Passive	RPLC	HILIC	UV–vis	[247]
Characterization of natural alkaloids	offline	Comp.	Manual	RPLC	SCX	UV–vis	[248]
Polar active compounds in curcuma kwangsiensis	offline	Comp.	Manual	RPLC	HILIC	MS	[249]
Prenylated phenolics in Glycyrrhiza uralensis	offline	mHC	Manual	NPLC	RPLC	ELSD, MS	[250]
Chemical constituents in Euphorbia kansui	offline	Comp.	Manual	NPLC	RPLC	MS, UV–vis	[251]
Profiling and comparison of ginseng	offline	Comp.	Manual	HILIC	RPLC	MS, UV–vis	[252]
Safflower methanol extract	online	Comp.	ACD	NPLC	RPLC	UV–vis	[253]
Differentiating root and rhizome ofpanax notoginseng	online	mHC	Passive	RPLC	RPLC	CAD, MS	[254]
Polyphenol compounds in propolis	offline	mHC	Manual	RPLC	RPLC	MS	[255]
Multicomponents from Cuscuta chinensis	offline	Comp.	Manual	HILIC	RPLC	MS	[256]
Characterization of huanglian jiedu Decoction	offline	Comp.	Manual	HILIC	RPLC	MS, UV–vis	[257]
TCM (Arenaria kansuensis)	offline	HC	Manual	RPLC	RPLC	UV–vis	[258]
TCM (Flavonoid glycosides from Lobelia chinensis Lour)	offline	Comp.	Manual	RPLC	HILIC	MS	[259]
TCM (bufadienolides in Venenum Bufonis)	offline	Comp.	Manual	RPLC	SFC	UV–vis	[260]
TCM (ginsenosides in Panax notoginseng leaves)	online	Comp.	SPAM	HILIC	RPLC	MS	[261]
TCM (ginsenosides from white and red ginsengs)	online	Comp.	SPAM	HILIC	RPLC	MS	[262]

addition to other fundamental advances in 2D-LC [267–269]. In addition to the application-oriented papers discussed throughout this review, numerous papers have also been published in the last three years describing technical advances in multi-dimensional separation; several of these are focused on computer-aided method development and optimization for 2D-LC.

Model-driven optimization of LC separations has a long history, and is quite popular in industrial laboratories where commercially available optimization software is used to build and apply retention models [270]. Recently, Den Uijl et al. [271] systematically studied the use of scanning gradients combined with retention modelling for LC optimization. In this work, the possibility of using retention data obtained from fast ^{2}D separations to build retention models to guide the development of ^{1}D elution conditions (with the same stationary phase) was investigated. The authors found that doing so yielded inaccurate predictions, probably because it involves extrapolation to gradient slopes beyond the range of those involved in the initial data collection using 2D-LC conditions. Later, Den Uijl et al. [272] demonstrated that scanning-gradient

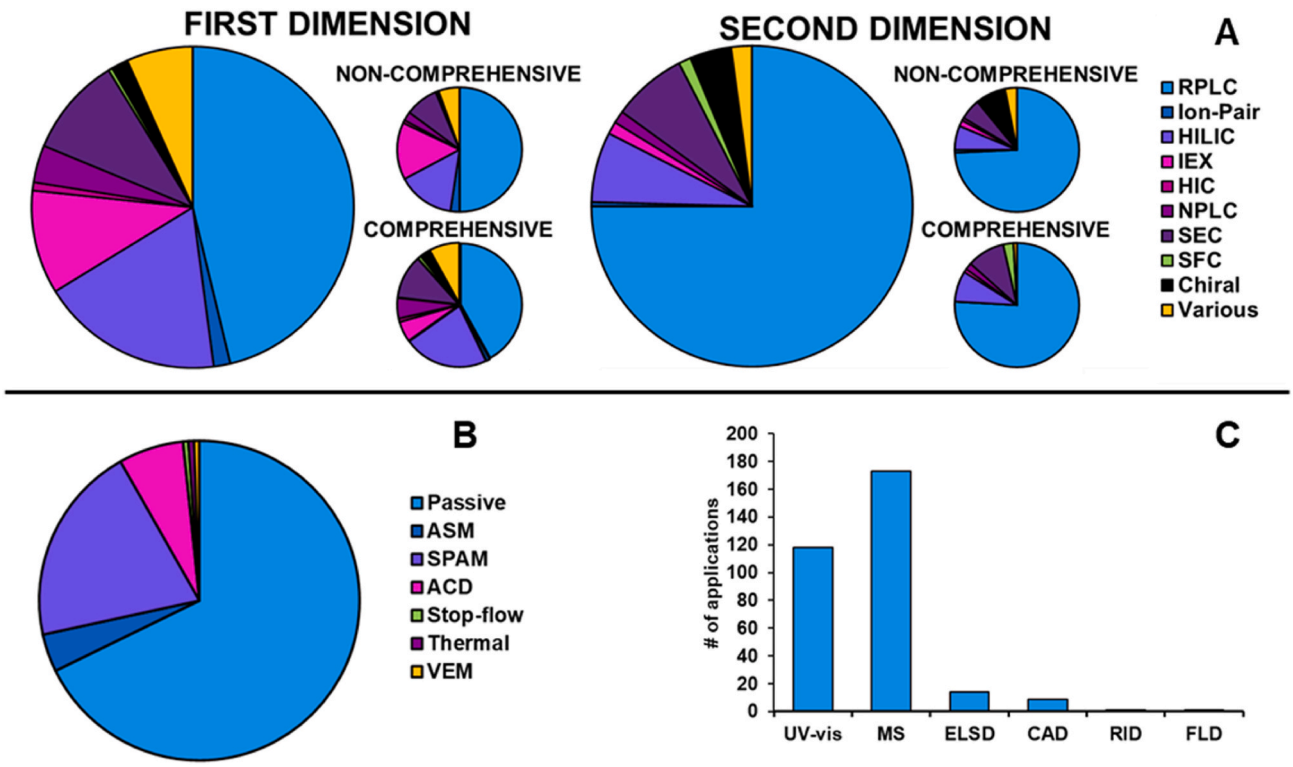

Fig. 1. (A) Overview of applied retention mechanisms in both the first (left) and second (right) dimensions with also distinction between comprehensive and non-comprehensive modes. (B) Used modulation strategies for all online applications. (C) Overview of applied detection techniques. Note that one application may use multiple detection techniques. Total number of applications: 240 (online comprehensive: 83, online non-comprehensive: 100, offline: 57). Data covers all applications from Table 1.

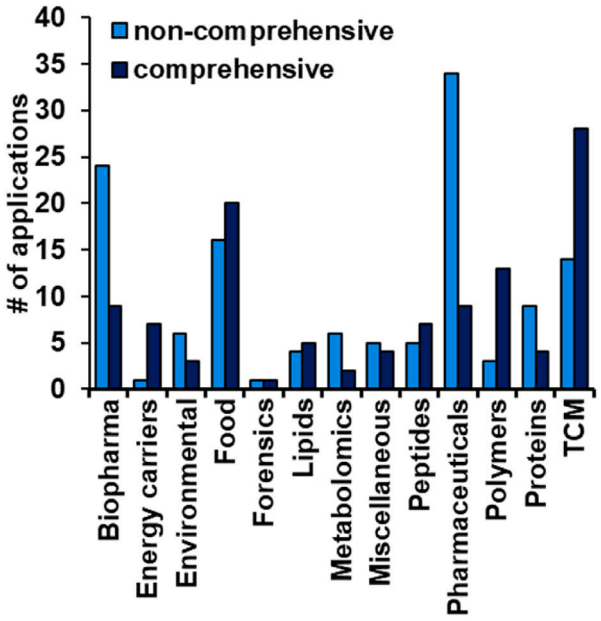

Fig. 2. Number of applications per application area distributed by non-comprehensive (light blue) and comprehensive (dark blue) applications.

based models can also be used to predict isocratic retention of analytes on trap columns under dilution-flow conditions used in SPAM. The authors demonstrated that the dilution flow should be selected carefully as under specific conditions, dilution with weaker eluent was demonstrated to be counterproductive. In other work, Boelrijk et al. [273] demonstrated the use of Bayesian optimization for 2D-LC optimization of many parameters with a limited number of experiments. Bayesian optimization outperformed grid searching and random search algorithms and is considerably more sample-efficient. Molenaar et al. [274] developed a peak-tracking algorithm for use in LC × LC and applied it to peptide retention data from varying chromatographic conditions for use in retention modelling. Makey et al. [275] and Haidar Ahmad et al. [163] applied retention modelling for streamlined 2D-LC method development in both chiral and achiral separations of pharmaceuticals. In these studies ^{2}D retention models were trained using retention data from either 1D-LC experiments, or the second dimension of 2D-LC experiments. The resulting models were then used to develop resolution maps dependent on variables such as column temperature and mobile phase composition, in a way similar to what has been done for 1D-LC for many years. The resulting resolution maps can be used to pinpoint the conditions that are likely to yield sufficient resolution of critical peak pairs in the second dimension. Stoll and Pirok [267] recently presented a brief overview of model-driven method development for 2D-LC. They concluded that retention modelling can reduce the amount of trial-and-error that is typically required. Indeed, this was then demonstrated by Stoll et al. [276,277] in the context of developing 2D-LC-MS methods for the discovery of impurities coeluting with the main peak in 1D-LC analyses of therapeutic peptides. In this work, retention data from ^{2}D separations were used to build a retention model that was used in turn to predict very shallow ^{2}D gradients needed to resolve closely related impurities eluting from the ^{1}D column.

3.2. Studies of other fundamental aspects of 2D-LC

3.2.1. Modulation strategies

In addition to predicting elution conditions, modelling has also been applied to investigate modulation strategies. One study investigated analyte breakthrough and simulated elution profiles of analytes from a 2D-LC loop-based modulation interface [278]. Moussa et al. [279]

studied radial dispersion in coiled loops to overcome breakthrough in high loop fillings compared to straight-capillary loops. Additionally, they studied analyte profiles and band broadening as a result of loops used in 2D-LC [280]. Recently in 2023, Knol et al. [281] demonstrated the use of packed modulation loops that promote radial dispersion similar to coiled loops which significantly reduces band broadening, particularly when using large loop volumes. They suggested that this strategy may also aid in preventing overfilling of the loops. Another study simulated elution profiles from modulation loops while using ASM at varied ^{2}D flow rates and loop sizes [282]. An even more recent study describes an optimization process for transfer and dilution when using ASM [283].

3.2.2. Dispersion in post-column flow splitting

In addition to the publications focused on method development and optimization, papers describing work on several other fundamental aspects of 2D-LC have also appeared in the last few years. One example is the examination of post-column flow splitting, which is often used for coupling high-flow rate ^{2}D separations to MS detection. Gunnarson et al. [284] demonstrated that flow splitting significantly reduces the peak volume, which is then more susceptible to dispersion (peak broadening) compared to situations where no splitting was applied. This highlighted the importance of carefully selecting the split ratio and dimensions of post-split tubing.

3.2.3. Data analysis approaches

Further improvements with regards to coupling 2D-LC with MS detection have been made in the area of data analysis as demonstrated by Pérez-Cova et al. [285]. Their work demonstrated that the combination of UV and MS detection with multivariate curve resolution increased the reliability of compound identification in the analysis of mixtures of pharmaceuticals. An in-depth review about the use of multivariate curve resolution in 2D-LC data has been published elsewhere [286]. Moreover, Molenaar et al. [287] developed an automated feature-mining algorithm for LC × LC-MS data from polymer separations using a mass-remainder analysis approach. The mass-remainder domain enabled distinction of polymer series with similar chromatographic behavior while series with similar mass-remainders were separated in the chromatographic domain. For other advances in chemometrics related to chromatography, the reader is referred to an exhaustive review that was recently published by Bos et al. [288].

3.2.4. 2D-LC with constant pressure operation in the second dimension

In other work related to instrumentation for 2D-LC, Shoykhet et al. [289] investigated the feasibility of operating the second dimension of a 2D-LC system under constant-pressure conditions. In this case, the flow rate is adjusted to compensate for mobile phase viscosity changes that occur during gradient elution to maintain a constant pressure at the column inlet, and enable full utilization of the pressure capabilities of the instrument. Additionally, operating at constant pressure reduces physical stress on ^{2}D columns with fast gradients which may result in longer column lifetimes.

3.2.5. Use of unconventional mobile and stationary phases

Aly et al. [116,290] demonstrated the use of "green" solvents for RPLC × RPLC separations. In their work, complementary separation mechanisms were achieved by using a mixture of propylene carbonate and ethanol as ^{1}D modifier and ethanol as ^{2}D modifier. Tang et al. [11] systematically demonstrated a method to improve the way solvent incompatibility between the first- and second-dimension separation can be handled. They showed that in some cases, the dilution that ASM offers can be insufficient, or that one might not have ASM instrumentation available. A relatively simple solution was demonstrated by use of a mixer in the modulation system, or a mixer in conjunction with ASM, to broaden and dilute even further the ^{1}D effluent profile to further prevent breakthrough. Around the same time, van den Hurk et al. [119] utilized a mixer in conjunction with SPAM to tackle the same issue and also enable large-volume (600 μL THF) heart-cut SEC-RPLC. The feasibility of using HILIC separations in the second dimension of 2D-LC has also been investigated further. Two studies demonstrated the use of repeatable partial equilibration in HILIC which improved the usability of HILIC as fast ^{2}D separation for comprehensive 2D-LC applications [291,292]. HILIC was also used as ^{2}D in a study demonstrating segmented 2D-LC using spatial RPLC as ^{1}D and time-based HILIC as ^{2}D [293].

3.2.6. Online multi-stage analysis involving analyte transformation

Finally, some advances have been made with regards to sample transformation in between separation dimension; this concept was also discussed in the book of Stoll and Carr [6]. Particularly notable is the recent proliferation of a family of applications aimed at streamlining the characterization of therapeutic proteins (monoclonal antibodies and antibody-drug conjugates) using multi-stage, multi-dimensional separation coupled with mass spectrometry. The emergence of this subset of applications began with the work of Gstöttner et al. [294–296] and has continued mainly with the work of a group comprised of Camperi, Goyon, and coworkers [19,21,297–302]. Camperi et al. [22] have recently reviewed much of the work in this area. Fig. 3 illustrates the general idea of multi-stage, multi-dimensional separation, where the first and last stages are LC separations (*e.g.*, Protein A affinity (ProA), IEX, HILIC, RP), and one or more of the intermediate stages involves a sample transformation (*e.g.* chemical reduction, enzymatic digestion). The terminal separation stage is usually HILIC or RP, due to their compatibility with MS detection. A major driving force for the development of these applications is the potential to combine many sample treatment and separation steps that traditionally have been executed serially offline into a series of steps executed serially, online, in a single instrumental method. This move from offline to online workflows reduces analytical variability, sample processing artifacts, analysis time, and human labor needed to acquire results. Such integration of analytical operations also enables process monitoring for production of biopharmaceuticals in new and exciting ways [21,300]. Most of the published work related to these applications has been driven by pharmaceutical companies and the industrial collaboration in this space will be further discussed further in the next section.

Besides therapeutic proteins, online intermediate sample transformation of guide RNAs was demonstrated by Goyon et al. [304]. The setup implemented an immobilized-RNase cartridge in between two separation dimensions enabling automated digestion and sequencing of an RNA molecule to gain insights on possible impurities. Sample transformation was also applied for photodegradation studies. Den Uijl et al. [145] incorporated a liquid-core-waveguide cell in a RPLC-RPLC system to enable fast photodegradation in between two separation dimensions. It was used to study intact dyes in the first dimension and subsequently perform photodegradation on selected heart-cuts, then the photodegradation products of that particular ^{1}D fraction could be studied in a ^{2}D separation. In a later study by the same group, the system was reconfigured to enable recycling by only using one column and re-injecting the photodegraded fraction onto the same column for separation of the degradation products [305]. This enabled repeated cycles of heart-cutting followed by photodegradation and analysis of the photoproducts to precisely elucidate photodegradation pathways; this approach can be thought of as LC^{n} in a way that is similar conceptually to MS^{n} in mass spectrometry.

Based on these technical changes, we have revised our earlier graphical overview of potential combinations of retention mechanism [3]. Table 2 reflects the strengths and weaknesses of potential combinations by a color gradient from red to green where dark green is the highest score. An overview of all the scoring parameters is presented in Supplementary Table 1.

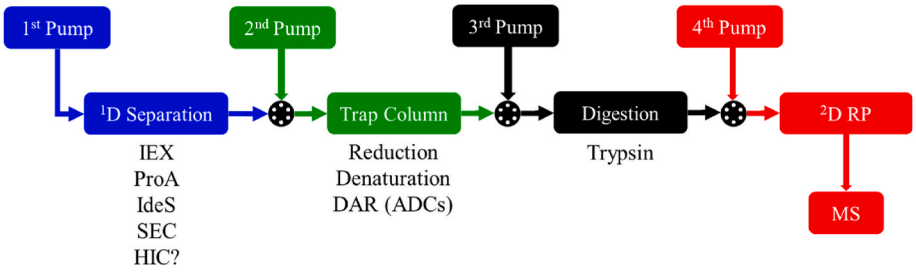

Fig. 3. Generalized illustration of a multi-stage, multi-dimensional approach for automated, online characterization of protein therapeutics. Adapted from Ref. [303].

Table 2
Overview of the possible online LC × LC combinations. The following abbreviations are used in the table - selectivity (S), breakthrough (B), adsorption (A). Fast separation (F), high resolving power (H), MS compatible (M), post-method equilibration (Q), orthogonality (O), mobile-phase compatibility (X), practicality (P), easy active modulation (E), isocratic (I). Superscript + or – indicates a positive or negative score for that parameter. Icons are used to indicate compatibility with polymers and/or proteins, reversing order, and to indicate a combination is published (see also Table S1). Adapted from Ref. [3].

	^{2}RP	^{2}NP	^{2}HILIC	^{2}HIC	^{2}IEX	^{2}SEC-Aq	^{2}SEC-Or	^{2}Ag	^{2}Chiral	^{2}Affinity	^{2}SFC
	$F^{2+}H^{+}Q^{+}M^{+}$	$F^{-}Q^{-}$	$M^{+}Q^{+}F^{2+}$	$F^{-}H^{-}M^{-}Q^{-}$	$M^{-}Q^{-}S^{+}$	$F^{+}H^{-}I$	$F^{+}H^{-}I$	$F^{-}Q^{-}S^{+}$	$F^{+}I\,S^{+}$	$H^{-}Q^{-}S^{+}$	$F^{+}H^{+}M^{+}$
^{1}RP H^{2+}	E O^{+} P^{+} X^{+}	B O^{2+} X^{2-}	B O^{2+} X^{+}	B E O^{-} P^{-} X^{+}	O^{+}	A E O^{+} P^{+} X^{+}	A E O^{+}	B O^{2+} X^{-}	O^{2+}	O^{2+} X^{+}	B O^{2+} X^{-} E^{-}
^{1}NP H^{+}	B O^{2+} X^{2-}	O^{-} P^{-} X^{+}	O^{-} P^{-} X^{-}	B O^{2+} P^{-} X^{2-}	O^{2+}	O^{2+} X^{2-}	O^{2+} P^{+} X^{+}	O^{+} X^{+}	O^{2+}	O^{+} X^{2-}	O^{-} X^{2+} E^{-}
^{1}HILIC H^{+}	B O^{2+} P^{+} X^{+}	B O^{-} X^{-}	O^{-} X^{+}	B O^{2+} P^{-} X^{-}	O^{+} X^{+}	O^{2+} P^{+}	A O^{+} X^{+}	B O^{+} X^{-}	O^{2+}	X^{-}	X^{+} E^{-}
^{1}HIC H^{-}	E O^{-} X^{2+}	B O^{2+} P^{-} X^{2-}	B O^{2+} X^{-}	O^{2-} P^{2-}	B O^{+} P^{-} X^{2+}	O^{2+} P^{-} X^{2+}	A O^{+} P^{-} X^{-}	B O^{2+} P^{-} X^{2-}	O^{2+} P^{2-}	O^{+} X^{+}	O^{+} P^{2-} X^{2-} E^{-}
^{1}IEX $H^{-}S^{+}$	E O^{+} P^{+} X^{2+}	B O^{2+} X^{2-}	B O^{+} X^{-}	B O^{+} P^{-} X^{2+}	B X^{-}	O^{+} X^{2+}	A O^{+} P^{+} X^{-}	B O^{+} X^{-}	O^{2+}	O^{+} X^{+}	O^{+} X^{2-} E^{-}
^{1}SEC-Aq H^{2-}	E O^{+} P^{+} X^{2+}	B O^{2+} X^{2-}	B O^{2+} X^{-}	B O^{+} P^{-}	O^{+} X^{2+}	O^{2-} P^{2-}	A O^{2-} P^{2-} X^{2-}	O^{2-} X^{2-}	O^{2+} P^{-}	O^{2+} X^{+}	O^{2+} P^{-} X^{2-} E^{-}
^{1}SEC-Or H^{2-}	B^{-} O^{+} X^{-} P^{+}	B O^{2+} X^{+}	O^{+} X^{+}	B O^{+} P^{-} X^{2-}	B O^{+} P^{-} X^{-}	O^{2-} P^{2-} X^{-}	O^{2-} P^{2-}	O^{2+} X^{+}	O^{2-} P^{-}	O^{2+} P^{2-} X^{-}	O^{+} P^{-} X^{+}
^{1}Ag $H^{+}S^{+}$	B O^{2+}	O^{+} X^{+}	O^{+} X^{+}	B O^{2+} P^{-} X^{-}	O^{2+} X^{-}	O^{2+} X^{-}	O^{2+} X^{-}	O^{2-} P^{2-}	O^{2+} P^{2-}	O^{2+} X^{2-}	O^{+} X^{+}
^{1}Chiral I S^{+}	O^{2+}	O^{2+}	O^{2+}	O^{2+} P^{2-}	O^{2+}	O^{2+} P^{-}	O^{2+} P^{-}	O^{2+}	O^{2-} P^{2-}	O^{2+}	O^{+} E^{-}
^{1}Affinity $H^{-}S^{+}$	O^{2+} P^{-} X^{+}	B O^{2+} P^{-} X^{-}	B O^{2+} P^{-}	O^{2+} P^{-}	O^{+} P^{-} X^{+}	O^{2+} P^{-} X^{+}	A O^{2+} P^{2-} X^{2-}	B O^{2+} P^{-} X^{-}	O^{2+} P^{-}	O^{-} P^{2-}	O^{+} P^{-} X^{2-} E^{-}
^{1}SFC H^{+}	E O^{2+} X^{+}	O^{-} X^{+}	E O^{-}	O^{2+} P^{3-}	O^{2+} X^{+}	O^{2+} P^{2-} X^{2+}	O^{2+} X^{2+}	O^{+} X^{+}	O^{2+}	O^{2+} X^{-}	E O^{-} X^{2+}

4. Multi-dimensional separations in industry

While there have been many technical advances in the field of 2D-LC in the past decade, most application papers are still published by academic research groups and there is, with some exception, little quantifiable evidence of routine use in industrial labs. Apart from confidentiality reasons, our view is that this is due to complexity of instrumental operation, extensive method development time, a small base of experienced users, and somewhat lower robustness of these complex systems relative to conventional 1D-LC systems. However, there is significant interest from industry to apply these novel separation systems, particularly in some specific application areas. To assess this, all the above-mentioned papers were screened on their author affiliations. It should be noted that government-funded research institutes and research hospitals were not labelled as "industry". For these 228 papers, 21.7% of all authors had an industry affiliation and 35.5% of the papers had at least one industry-affiliated author. The total number of applications and the number of applications with industry involvement were examined from the data in Table 1. Fig. 4 shows the percentage of all published applications that involved at least one industry-affiliated author, organized by application area.

Fig. 4 shows that the degree of industry involvement is not evenly distributed across the application areas. It is noteworthy that there have been no published applications focused on proteins with industry-affiliated authors. There has been some industry involvement in peptide-oriented applications, but the rate is low at less than 10%. The areas with the highest levels of industry involvement polymers (13 of 16), biopharma (21 of 33), and pharmaceuticals (22 of 43) with >50% in each case, indicating strong industry knowledge and even technical leadership in these areas. It should also be noted that authors affiliated with instrument manufacturers were also involved in many of these recent papers (each of four different major manufacturers was involved in two to six papers). Additionally, 92% of the industry-affiliated applications describe online methods, while only 76% of all described applications are online, indicating an industry preference for online 2D-LC approaches. This is also what is expected from an industrial perspective where reduced manual labor is preferred due to the high cost of personnel and due to longer time offline approaches may require. Furthermore, comprehensive separation is the most prominent mode for polymers, which makes sense based on the many structural distributions present in these materials. On the other hand, heart-cutting approaches are very often used in pharma and biopharma as the targeted applications.

Additional interesting trends are found when we look at both the usage of specific retention mechanisms, and industry-affiliated work. First, industry-affiliated authors were involved in most of the

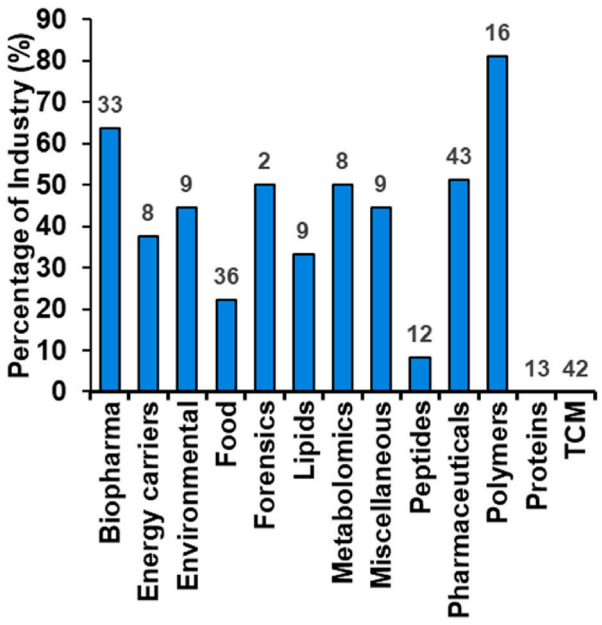

Fig. 4. Percentage of applications per field in which at least one of the authors was affiliated with industry. The total number of applications per field (both Industry and non-industry) is displayed on top of each bar.

applications where NPLC was used in either the first or the second dimension; NPLC is frequently used for separations of polymers and lipids. Moreover, SEC is a common separation mechanism for polymers and it was the second most popular (after RPLC) separation mechanism used in the industry-affiliated applications. When examining applications involving chiral separations, industry was involved in 9 of the 13 applications. As mentioned above, industry involvement in the field of pharmaceuticals was significant, which is where most activity in chiral separations lies. Other commonly used mechanisms are RPLC, HILIC, and IEX. However, for all these, roughly one in three applications have an industry affiliation, which is the same fraction found for industry involvement in all applications. This suggests that there is no specific industry preference for use of these mechanisms, while there is a positive bias for use of SEC, NPLC, and chiral separations in industrial labs.

With regards to detection, ELSD is an uncommon detection method, but very useful for non-volatile molecules without chromophores. In fact, 12 of the 14 ELSD applications were in the polymer field, for which MS is rarely applicable. Additionally, 6 of the 9 applications with CAD as detection method were industry affiliated. For the most common detection methods, about 40% of applications involving UV–Vis detection were industry-affiliated, while about 34% (58 of 173) involving MS detection were industry-affiliated. These rates are similar to the overall rate of industry involvement in all published applications, suggesting that there is not a strong bias for either technique in industry. This is quite interesting considering that the high cost and complexity, and lack of robustness with high-end MS systems are often discussed as factors preventing wider adoption in industrial labs. It should also be noted that some industrial research involving higher-end MS may not be publishable for confidentiality reasons.

In recent years there has been considerable interest in the idea of "universal 2D-LC methods" that would require minimal adjustment by non-expert users before applying them to real application problems [4]. Although such methods have not emerged broadly, one area where we are seeing this concept have a big impact is in the characterization of protein therapeutics using the multi-stage, multi-dimensional methods discussed above in Section 3.2.6. A widespread perception that such methods are easy to use, robust, and applicable to a variety of protein targets will accelerate adoption of these methodologies. To this end, Camperi et al. [20] have coordinated in inter-laboratory study to compare the results from CEX-reduction-digestion-RP-MS methods for the characterization of monoclonal antibodies. The study included an offline method, and online methods executed in three different laboratories (on different continents) and with instrument hardware and software from different vendors. The authors found that online methods produced results comparable to the offline method, and that the results from the three different laboratories were comparable in terms of important performance metrics including amino acid sequence coverage and abundance of specific post-translational modifications. This is a landmark result that should increase confidence of prospective 2D-LC users to make the leap and consider wider adoption of 2D-LC methods.

5. Concluding remarks

2D-LC continues to gain popularity as seen from the over two hundred articles published in the last four years that make use of some form of 2D liquid-based separation, in many different application areas. These articles were compared to those from earlier years that were reviewed previously in 2019 to look for trends in the field. In addition to articles that describe new applications of 2D-LC, significant technical advances have also been made in the past few years. One of the focal points of technical development continues to be mobile phase mismatch when combining complementary retention mechanisms. In this area, we discussed the rise of At-Column Dilution (ACD) as an active modulation strategy, in addition to the already popular options of SPAM and ASM to achieve peak focusing. The importance of mixers in modulation strategies has also been highlighted. SPAM continues to be the most popular active modulation strategy, and recent work has shown that retention modelling can be used to further improve and optimize SPAM parameters. Regarding computer-aided method development, another area of emphasis where research aims streamline method development and reduce complexity of 2D-LC for implementation in industrial environments, several articles described promising results. Several new chemometric approaches that aid with the analysis of MS data have been published; this activity is important given that MS was the most used detection method in recently published 2D-LC applications.

To assess the extent of industry involvement in recent 2D-LC development and applications, author affiliations were studied. In fact, over 34% of the >200 papers discussed had at least one author that was affiliated with industry. Additional trends were observed that highlighted industry's interest, particularly in the fields including polymer analysis, metabolomics, and pharmaceutical and biopharmaceutical analysis. Industry laboratories are also using more online than offline 2D-LC applications, which makes sense from the perspective of reducing the manual labor involved in the analyses. In some cases, online multidimensional separations can be utilized to eliminate certain laborious sample preparation steps; heartcutting 2D-LC has been particularly popular for this purpose. It should be highlighted again that there may be more 2D-LC implementations in industrial laboratories that are not published for confidentiality reasons.

The large volume of recent applications of 2D-LC combined with the significant involvement of industry in the development of new applications demonstrates that there is a large interest in improving and implementing 2D-LC for various modern applications that require multidimensional separation. We expect that continued work on computer-aided strategies to support method development, along with instrumental improvements to increase robustness of these complex systems will lead to more implementation of 2D-LC methods in industrial laboratories in the coming years. A recent inter-laboratory comparison study of the performance of a multi-stage, multi-dimensional separation for characterization of protein therapeutics is increasing confidence that this type of method can be deployed more broadly. We hope to see more studies of this type in the years to come. The volume and diversity of research in the field discussed in this review suggests a bright future for the use of 2D-LC.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.trac.2023.117166.

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