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Simultaneous analysis of various anticancer drugs by supercritical fluid chromatography-mass spectrometry. Part I: Optimization of chromatographic separation

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ABSTRACT

This work presents a generic SFC-MS method for the simultaneous analysis of 22 anticancer drugs (fluorouracil, busulfan, cyclophosphamide, cytarabine, dacarbazine, daunorubicin, docetaxel, doxorubicin, epirubicin, etoposide, gemcitabine, idarubicin, ifosfamide, irinotecan, methotrexate, paclitaxel, pemetrexed, raltitrexed, topotecan, treosulfan, vinblastine, vincristine). The separation conditions were optimized by screening nine stationary phases (2-picolylamine, bare hybrid silica, 2-ethylpyridine, fluoro-phenyl, octadecyl, diethylamine, diol, 1-aminoanthracene, zwitterionic modification), evaluating additives effects (2–5 % water, 20–50 mM ammonium formate, 0–1 mM ammonium fluoride), and adjusting the organic modifier composition (methanol, ethanol, isopropanol, acetonitrile). The optimized SFC-MS method successfully analyzed 22 anticancer drugs, along with 5 additional challenging compounds (azacitidine, mitomycin, cisplatin, oxaliplatin, carboplatin), in 12 min, using a diol column (100 × 3 mm, 1.7 μm) and a gradient of 2–100 % methanol containing 2 % water and 50 mM ammonium formate. To overcome overpressure generated by high organic solvent content, a backpressure gradient (110–150 bar) and a flow rate gradient (0.6–1.5 mL/min) were applied. The diol column was selected as the most promising based on five predefined chromatographic criteria. Additives with 5 % water or ammonium fluoride were excluded due to overpressure and signal loss, respectively. Increasing ammonium formate concentration improved peak symmetry by 29 %. For the organic modifier, pure methanol was chosen since ternary mixtures led to system overpressure without improving separation. Comparison with the LC-MS method using real samples confirmed the potential applicability of the SFC method, as the same trace compounds were detected with comparable concentrations. Sensitivity optimization and method validation will be discussed separately in a later paper.

1. Introduction

Conventional anticancer drugs (CADs) are highly reactive and toxic active ingredients. Due to their carcinogenic, mutagenic and reprotoxic effects, they are classified as CMR substances. For patients undergoing treatment, these drugs can cause a range of side effects, such as nausea, vomiting, hair loss, and myelosuppression. In a hospital context, healthcare professionals can also face regular exposure to CADs throughout the drug life cycle, from receipt to waste disposal. This recurring exposure could represent a health risk to various workers, including pharmacists, nurses, technicians, and cleaning staff. Reported risks associated with exposure to CADs include genotoxic effects and adverse impact on development and reproduction [1]. To monitor workers' exposure and ensure their workplace safety, it is possible to implement environmental monitoring, such as surface contamination analysis, or/and the detection of CADs in biological samples from potentially exposed healthcare professionals.

Given their diverse physicochemical properties, analytical

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techniques for quantifying these CADs, especially at trace levels in environmental or biological samples, must be both highly sensitive and selective. Liquid chromatography coupled to mass spectrometry (LC-MS) is the technique of choice for this purpose, offering excellent sensitivity and selectivity. Numerous LC-MS methods have been developed for the analysis of multiple CADs in environmental [2,3] and biological matrices [4,5]. However, LC-MS faces challenges when analyzing compounds with highly diverse physicochemical properties, as is the case with CADs. Indeed, reverse-phase liquid chromatography (RPLC) is more suitable for hydrophobic compounds, whereas normal phase is more adapted for polar compounds. Developing a single method capable of simultaneously identifying and quantifying various drugs, without sacrificing the ability to analyze highly polar or hydrophobic compounds, present a significant challenge. Additionally, some CADs are unstable in aqueous conditions, making their analysis difficult under conventional RPLC conditions. For example, cisplatin exists in equilibrium with its complex forms in water [6], and azacitidine undergoes hydrolysis in aqueous solution [7].

In this context, Supercritical Fluid Chromatography (SFC) emerges as a promising alternative for separating a wide range of compounds. Its unique characteristics allow for the separation of compounds with diverse physicochemical properties within a single method, as shown in several publications in the fields of pesticides [8] or pharmaceuticals [9, 10]. The absence, or at least limited presence, of water in SFC may also facilitate the analysis of compounds that are unstable under aqueous conditions. For example, Wang et al. and Liu et al. successfully utilized SFC to analyze ginkgolides (extracts of Ginkgo biloba), avoiding the hydrolysis that typically occurs in RPLC with aqueous eluents [11].

The distinctive properties of SFC lies in the use of a supercritical fluid as the main mobile phase, enabling higher resolution efficiency and faster analysis [12]. Typically, the mobile phase is composed of carbon dioxide combined with an organic modifier to expand its polarity range. Small quantities of additives can also be incorporated to improve peak shapes for certain compounds [13]. The common gradient in SFC involved an increase from 2 % to 40 % organic modifier, but it has been extended to 100 %, a technique known as "sub/supercritical fluid chromatography," to further extend the mobile phase polarity range [13].

Concerning the analysis of CADs, SFC is scarcely used. Existing publications using SFC for CADs typically focus on the analysis of one to four compounds in various matrices. Most SFC-MS methods have been developed to analyze one or two CADs in plasma [14–16], pharmaceutical formulations [17,18] or wastewater samples [19]. To our knowledge, only two studies reported the simultaneous analysis of several CADs. For instance, Jin et al. evaluated three taxane drugs (paclitaxel, cabazitaxel, and docetaxel) in rat blood [20], while Musser and Callery analyzed four CADs (cyclophosphamide, diaziquone, mitomycin C and thiotepa) during their fundamental study of SFC-MS interface design under chemical ionization conditions [21].

The aim of this study was to evaluate the potential of SFC-MS/MS for the simultaneous detection and quantification of 22 CADs at trace levels. The evaluation of the chromatographic separation of target compounds is the main focus of this article. A generic SFC-MS/MS method was developed to identify all these compounds. For this purpose, a screening of several stationary phases and an evaluation of different mobile phase conditions (additive type and concentration, organic modifier composition) were performed. Once the SFC method was optimized, five additional challenging CADs known for their instability, were also analyzed.

2. Material and methods

2.1. Chemical, reagents and columns

All solvents used were MS grade, with the exception of dimethyl sulfoxide and ethanol. Formic acid and ammonia (25 %) were purchased from Merck (Darmstadt, Germany). Methanol (MeOH), ethanol (EtOH), isopropanol (IPA), acetonitrile (MeCN), dimethyl sulfoxide (DMSO), ammonium formate (NH_4HCO_2, $\geq$ 99.0 %) and ammonium fluoride (NH_4F, $\geq$ 99.99 %) were obtained from Sigma-Aldrich (Buchs, Switzerland). Ultrapure Type 1 water was produced using a Milli-Q purification system from Millipore (Bedford, MA, USA). Pressurized carbon dioxide (CO_2) 4.5 grade (99.995 %) was obtained from Linde Gas Schweiz AG (Dagmersellen, Switzerland).

All chemicals were of the highest available analytical quality. Busulfan, cisplatin, cytarabine, daunorubicin hydrochloride, doxorubicin hydrochloride, epirubicin hydrochloride, gemcitabine hydrochloride, methotrexate, mitomycin, oxaliplatin, paclitaxel, vinblastine sulfate and vincristine sulfate were purchased from European Pharmacopoeia (Strasbourg, France). 5-fluorouracil, azacitidine, carboplatin, cyclophosphamide, dacarbazine, docetaxel trihydrate, idarubicin, ifosfamide, irinotecan hydrochloride, pemetrexed, raltitrexed and treosulfan, were obtained from Sigma-Aldrich (Buchs, Switzerland). Etoposide and topotecan hydrochloride were purchased from Toronto Research Chemical (North York, Ontario, Canada).

Internal standards (stable isotope-labeled analogs) were obtained from several suppliers. [$^2H_8$]-Busulfan, [$^2H_8$]-Cyclophosphamide monohydrate, [$^{13}C_6$]-irinotecan, [^{13}C, $^2H_3$]-methotrexate, [$^2H_5$]-paclitaxel, [^{13}C, $^{15}N_2$]-5-fluorouracil were obtained from Alsachim (Strasbourg, France). [$^{13}C_3$]-Cytarabine and [$^2H_{10}$]-oxaliplatin were purchased from Toronto Research Chemical (Ontario, Canada).

Nine stationary phases were investigated. Acquity UPC^2 Torus 2-picolylamine (2-PIC), Acquity UPC^2 Torus High-density Diol (DIOL), Acquity UPC^2 Torus diethylamine (DEA), Acquity UPC^2 Torus 1-aminoanthracene (1-AA), Acquity UPC^2 HSS C18 SB (C18), Acquity UPC^2 Bridged Ethylene Hybrid (BEH), Acquity UPC^2 BEH 2-Ethyl-Pyridine (2-EP) and Acquity UPC^2 CSH Fluoro-Phenyl (FP) were obtained from Waters (Milford, MA, USA). Nucleoshell HILIC (HILIC) was purchased from Macherey-Nagel (Düren, Germany). All these columns had a length of 100 mm, an internal diameter of 3.0 mm and a particle size of 1.7 μm (or 1.8 μm) – except for HILIC column with a particle size of 2.7 μm. Detailed information on the columns is provided in Table 1.

2.2. Safety considerations for the handling of anticancer drugs

Due to the high toxicity of CADs, strict safety protocols were implemented to minimize exposure for analysts and their surroundings. All experiments involving these hazardous compounds were performed in a specialized laboratory, Cytoxlab.[1] This facility maintains negative pressure to effectively confine potential contamination. Toxic powders were weighed and dissolved within a safety weighing enclosure equipped with horizontal airflow and HEPA H14 filters (ST1 Safety Weighing Enclosure 1200, a1-enviroscIences GmbH, Düsseldorf, Germany). Most dilution steps were automated using a liquid handling workstation (Tecan Freedom EVO®, Männedorf, Switzerland). Instruments and materials that came into contact with toxic compounds were treated as hazardous waste. Personal protective equipment (PPE), including gloves, gowns, and masks, was selected in accordance with established guidelines from the literature [1].

2.3. SFC-MS/MS instrumentation

The SFC system used was the AQCUITY Ultra Performance Convergence Chromatography™ (UPC^2) from Waters Corporation (Millford, MA, USA). It was equipped with a binary solvent delivery pump (Binary Solvent Manager UPC^2), an autosampler (Sampler Manager UPC^2), a backpressure regulator (Convergence Manager UPC^2), a column oven (Column Manager UPC^2), and a sheath pump for make-up solvent

[1] More information on www.cytoxlab.ch, Gabrielle Perret-Gentil 4, 1211 Geneva, Switzerland

Table 1
Columns used in the study.

Abbreviation	Stationary Phase	Supplier	Brand	Dimension [mm]	Particle Size [µm]	Particle Technology	Support particle
1-AA	1-aminoanthracene	Waters	Torus	3.0 × 100	1.7	FPP	Hybrid
2-PIC	2-picolylamine	Waters	Torus	3.0 × 100	1.7	FPP	Hybrid
BEH	Bare hybrid silica	Waters	Viridis	3.0 × 100	1.7	FPP (BEH)	Hybrid
2-EP	2-ethylpyridine	Waters	Viridis	3.0 × 100	1.7	FPP (BEH)	Hybrid
FP	Fluoro-phenyl	Waters	Viridis	3.0 × 100	1.7	FPP (CSH)	Hybrid
DEA	Diethylamine	Waters	Torus	3.0 × 100	1.7	FPP	Hybrid
DIOL	1.2-dihydroxypropyl ester	Waters	Torus	3.0 × 100	1.7	FPP	Hybrid
C18	C18 SB	Waters	Acquity UPLC	3.0 × 100	1.8	FPP (HSS)	Silica
HILIC	Ammonium - sulfonic acid (zwitterionic) modification	Macherey-Nagel	Nucleoshell	3.0 × 100	2.7	SPP	Silica

SPP: Superficially Porous Particles; FPP: Fully Porous Particles.
BEH: Bridged Ethyl Hybrid; CSH: Charged Surface Hybrid; HSS: High Strength Silica.

(Isocratic Solvent Manager). The Back Pressure Regulator (BPR) heater system was set at 70 °C. The chromatographic system was connected to the mass spectrometer via a "pre-BPR splitter with sheath pump" interface, as detailed elsewhere [22]. The SFC system was controlled with Empower® 3 software. The samples were injected at 1 µL, with a concentration of 100 and 400 ng/mL. After each injection, the injector needle was washed with 200 µL IPA followed by 600 µL of a solution composed of MeCN/IPA/MeOH (7:2:1). The make-up solvent was pure MeOH pumped at 0.3 mL/min.

The mass spectrometer used was a triple quadrupole QTRAP 6500 + from AB SCIEX (Framingham, MA, USA) equipped with a Turbo Spray IonDrive source. It operated in MRM mode with an electrospray ionization (ESI) interface, utilizing fast polarity switching to enable the simultaneous detection of all target analytes in both positive and negative ionization modes within a single analytical run. The MS parameters of the compounds are documented in Table S1 in the supplementary material. Other MS source parameters included: ion spray voltage was 5000 V in positive and -2500V in negative mode; entrance potential was 10 V in positive and −10 in negative mode. For both modes, curtain gas was 25 psi, temperature was 350 °C, and ion source gas (GS1 and GS2) were 60 psi and 70 psi, respectively. Sciex® software was used for setting MS parameters and data acquisition, while Analyst® software was used for peak integration. All resolution values were calculated manually based on the peak widths measured at half the peak height by the software with the following formula:

$$R_s = 1.18 \quad x \quad \frac{t_{RB} - t_{RA}}{W_{0.5B} + W_{0.5A}}$$

where $W_{0.5}$ is the width of the peak at 50 % height, which was determined by the acquisition software.

To synchronize the instruments from different manufacturers, a custom cable was created. One end was connected to the AUX I/O port of the MS via an RS-232 connector, while the other end was wired to the contact closure signals of the autosampler in the SFC system.

2.4. Sample preparation

2.4.1. For optimization experiments

Stock solutions of 22 CADs, including 5-fluorouracil, busulfan, cyclophosphamide, cytarabine, dacarbazine, daunorubicin, docetaxel, doxorubin, epirubicin, etoposide, gemcitabine, idarubicin, ifosfamide, irinotecan, methotrexate, paclitaxel, pemetrexed, raltitrexed, topotecan, treosulfan, vinblastine and vincristine, were each dissolved in DMSO at a concentration of 1 mg/mL and stored at −80 °C until use. Table S2 from the supplementary material summarizes the main physico-chemical properties of all the CADs used in this study. A mixture of the 22 standards was freshly prepared in MeCN/water (1:1) at two different concentrations: 100 ng/mL and 400 ng/mL for each compound. The lower concentration was used for peak identification and the higher concentration was used for peak confirmation.

2.4.2. For preliminary validation experiments

To demonstrate the potential of the developed SFC-MS method, preliminary quantitative experiments were achieved. To estimate function responses and sensitivity, six standard samples at 0.5, 1, 5, 10, 50, 100 and 200 ng/mL were prepared. The low limit of quantification was determined for each CAD with an S/N higher than 10:1. Each standard sample contained the 27 CAD in a mixture of MeCN:water (1:1) and the eight internal standards (at 5 mg/mL except for [$^2H_{10}$]-oxaliplatin at 20 mg/mL). Accuracy and precision were determined with the analysis of 6 independent quantification samples prepared at 100 ng/mL. Accuracy was expressed as percentage, as the ratio between theoretical and average measured values. Precision was defined as the relative standard deviation (RSD). For all sequences, quality control samples at low 10 ng/mL and higher concentration 50 ng/mL were analyzed to ensure the reliability of the method.

2.4.3. For method application

To demonstrate the applicability of the developed SFC-MS method to real samples, analyses of the 27 CADs were performed with wiping samples for environmental monitoring. Several surfaces were evaluated in hospital pharmacy chemotherapy compounding units. The same wipe sampling procedure was applied for all locations [23].Surfaces were wiped with the moistened swab. After complete drying, the swab was desorbed in a mixture of MeCN/water (1:1) and the pool of the eight internal standards was added to obtain a final concentration of 5 ng/mL except for [$^2H_{10}$]-oxaliplatin (20 mg/mL).

3. Results and discussion

To optimize the chromatographic separation of CADs, three key steps were carried out. Initially, a generic gradient was established based on the range of compounds to be analyzed. The first step involved screening various stationary phases to identify the one that provided optimal separation for the CADs. Once the best column was selected, the second step focused on evaluating the impact of different additives, including their nature and concentration. Finally, the third step assessed various compositions of the organic modifier to determine their influence on the separation of CADs.

3.1. Selection of gradient conditions

Preliminary tests were conducted using a typical gradient ranging from 2 % to 40 % organic modifier at 40 °C, with the BPR set at 150 bar. Methanol supplemented with 2 % water and 20 mM NH_4HCO_2 was used as the organic modifier. These additives were systematically included in the mobile phase for several reasons: water increases the polarity and

solvation power of the mobile phase, facilitating the elution of highly polar analytes and improving peak shapes and efficiency [24]. NH_4HCO_2, a volatile salt compatible with MS, aids in the elution of polar and ionic compounds [25]. These initial tests were performed on two different stationary phases, BEH and 2-PIC, which have acidic and basic characteristics, respectively. The BEH column, which is a bare hybrid silica, is widely applied in SFC [26]. It is a generic column for analyzing all types of molecules, especially acidic and neutral compounds, or even basic compounds with additives. Whereas the 2-PIC column is highly suitable for analyzing basic compounds, even without additives, and is commonly used in pharmaceutical analysis [26]. The results indicated that some compounds were highly retained with this gradient: on the BEH and 2-PIC column, four (pemetrexed, doxorubicin, epirubicin, idarubicin) and three (raltitrexed, pemetrexed, methotrexate) analytes were not eluted, respectively. Additionally on the BEH column, four other CADs (topotecan, raltitrexed, methotrexate, irinotecan) exhibited late elution during the final isocratic segment at 40 % MeOH, likely due to the wide range of physicochemical properties of the compounds tested.

To address the strong retention of certain compounds, the gradient was modified by increasing the concentration of organic modifier up to 100 % to facilitate their elution from the column. This modification has been previously described in the literature for other compounds classes [27]. While this approach shows promise for eluting highly polar compounds, it presents the challenge of increased pressure due to the high proportion of organic modifier. Two solutions were implemented to address this issue. First, the flow rate was gradually reduced from 1.5 to 0.6 mL/min during the second half of the gradient (from 40 % to 100 % MeOH), despite increasing analysis time. Second, the backpressure was lowered during the analysis, ensuring it remained above the minimum threshold required for proper SFC system operation. It is important to note that backpressure has less impact on separation when the organic modifier concentration is between 40 % and 100 % MeOH. Consequently, a pressure ramp from 150 to 110 bar was introduced in the second half of the gradient to mitigate the pressure increase. The gradient slope was also tuned to achieve the best compromise between chromatographic performance and analysis time.

The influence of temperature was not investigated, despite its potential role in the separation of the CADs, for several reasons. Frist, increasing the temperature to 60°C (instead of the usual 40°C) led to stability issues, such as the degradation of several compounds. Secondly, one of the most challenging separations was the epimeric pair, which eluted at around 40 % methanol. At such a high organic solvent content, the effect of temperature becomes less significant, as the organic solvent dominates the mobile phase composition, thereby reducing the influence of CO_2 density changes.

Finally, in the generic gradient used for method development, mobile phase B was composed of MeOH with 2 % water and 20 mM ammonium formate. The gradient was defined as follows: an initial mobile phase composition of 98 % CO_2/2 % MeOH was kept constant for 1 min, followed by a ramp up to 40 % B over 5 min at a flow rate of 1.5 mL/min and a backpressure of 150 bar. Then, a second ramp increased the organic modifier to 100 % B at 10 min with the flow rate reduced to 0.6 mL/min and the backpressure lowered to 110 bar. The gradient returns to its initial conditions in 0.1 min until 12 min (end of the analysis). This gradient was used for all subsequent experiments.

3.2. Screening of stationary phases

The evaluation of various stationary phases was conducted using the previously defined gradient conditions. Nine different phase chemistry, listed in Table 1, were tested, based on the recent work of West et al. [28]. These stationary phases were selected for their complementary selectivity, derived from their distinct chemical properties, as shown in Fig. 1. The DIOL column is neutral and highly polar, while the 1-aminoanthracene (1-AA) is slightly basic, capable of strong π-π interactions and shape recognition. The Bridged Ethylene Hybrid (BEH) is the most acidic, with the presence of numerous silanol groups. The 2-Ethyl-Pyridine (2-EP) and 2-picolylamine (2-PIC) columns share a basic nature and have similar chemical characteristics. The diethylamine (DEA) column, also basic, lacks aromatic rings in its bonding, making it, along with the other basic columns, ideal for analyzing basic molecules. The Fluoro-Phenyl (FP) column has a positive surface charge (surface charge hybrid) and can create various interactions with analytes, such as H-bonding, dipole-dipole, π-π interactions, and shape recognition. The non-endcapped C18 column has numerous accessible silanol groups, responsible for its polar characteristics. Lastly, the HILIC stationary phase present zwitterionic properties, with both ammonium and sulfonic acid groups in its structure.

The performance of each column was evaluated using five key criteria: (1) the number of compounds successfully eluted with the generic gradient; (2) the separation of two critical isomeric pairs with identical or nearly identical mass-to-charge ratios for precursor ions (epirubicin/doxorubicin and cyclophosphamide/ifosfamide); (3) the number of co-eluting compounds; (4) the average asymmetry factor of all compounds, calculated at 10 % of peak height; and (5) the retention

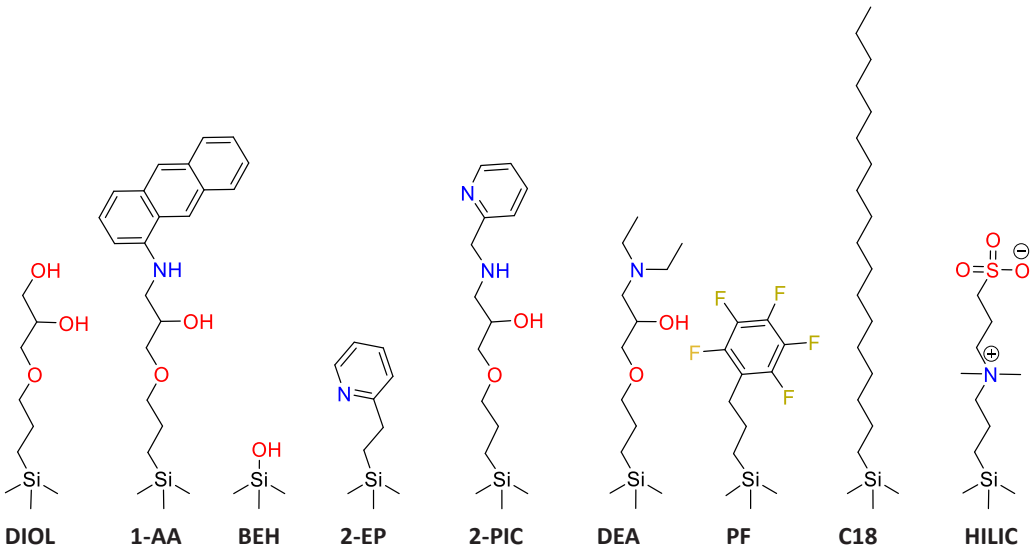

Fig. 1. Chemical structure of stationary phases tested.

time of the last eluted compound. Table S3 from the supplementary material provides details on the determination of the five criteria used to evaluate each stationary phase, along with the scoring system ranging from 1 (worst) to 5 (best). The column with the highest total score was chosen for further evaluation. Fig. 2 presents a radar plot visualizing the distribution of scores across the five criteria for each tested stationary phase. As shown, the radar plot areas varied significantly between columns. The DIOL column emerges as the most promising, with a score of 21 points, followed by C18 with 19 points and HILIC with 17 points. In contrast, the 2-EP and the FP columns delivered suboptimal performance, scoring 13 and 10 points, respectively. The following discussion focuses on the main observations for the five criteria across the nine columns.

The first criterion was the number of detected peaks. Out of the nine stationary phases, only six (2-PIC, DIOL, BEH, 2-EP, C18 and HILIC) allowed the elution of all compounds. Some substances were too strongly retained on the other three columns and could not be detected, even with a gradient ranging from 2 % to 100 % MeOH. For example, the four anthracycline molecules (doxorubicine, epirubicin, daunorubicin and idarubicin) were not eluted on the 1-AA column. This is likely because this stationary phase can form numerous π-π interactions with the anthracycline molecules, which contain an anthracene-like aromatic group rich in π electrons. In addition, these molecules have acidic phenol groups that may interact electrostatically with the amino group on the 1-AA bonding. Besides the anthracyclines, pemetrexed and methotrexate were also problematic, as they could not be eluted from the DEA column. At the working pH (around pH 4–5, with supercritical fluid CO_2 and MeOH mixture [13]), the DEA column surface carried a positive charge, resulting in strong anion exchange interactions. This likely caused strong retention of these two compounds, which each have two carboxylic acid groups on their structures that are negatively charged at this pH. Interestingly, methotrexate was also not eluted from the FP column under the mobile phase conditions used in this study. This may be attributed to the fluorophenyl group acting as a strong Lewis acid,

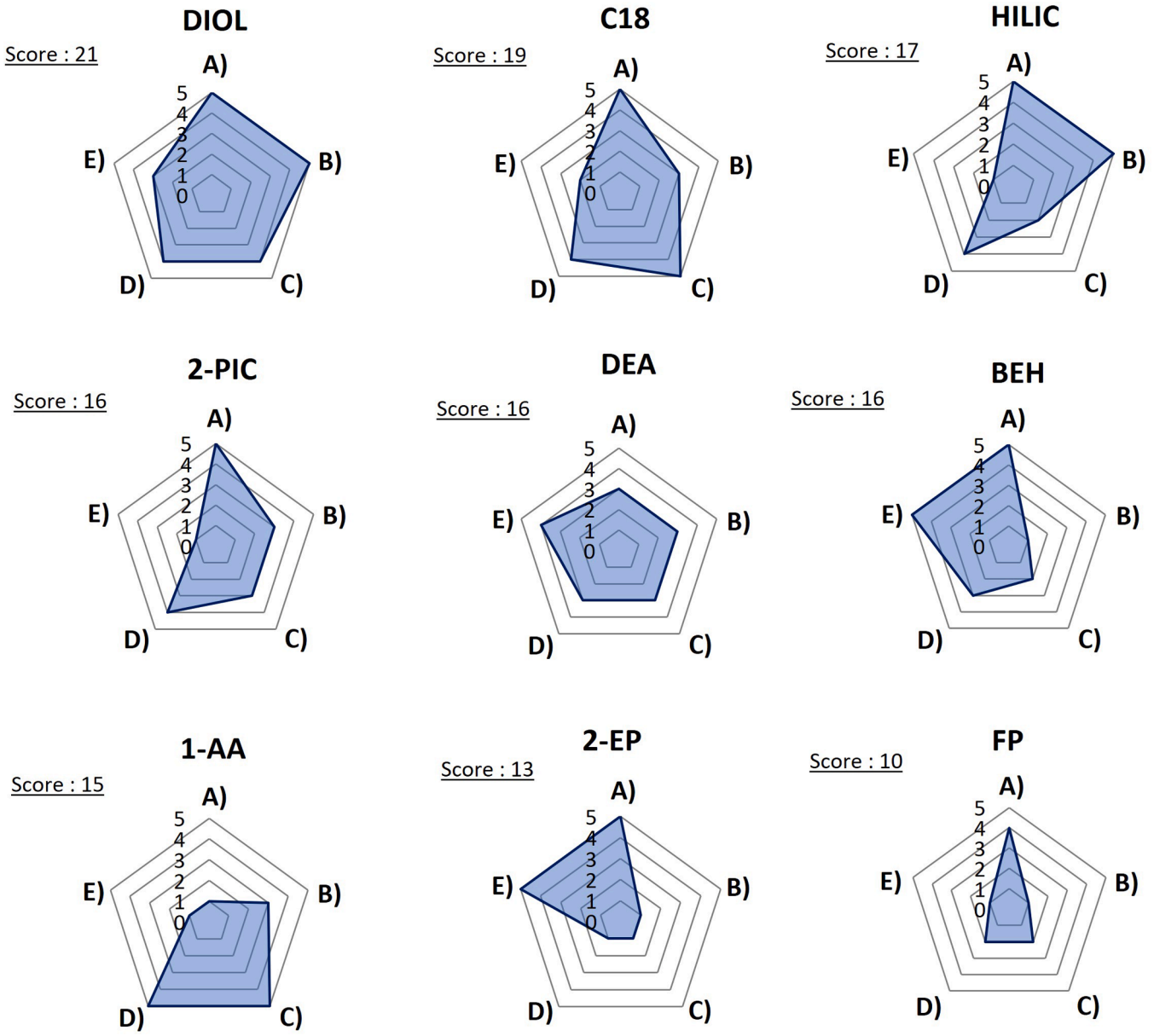

Fig. 2. Radar plots of the nine stationary phases evaluated. The five criteria used to score each column are: A) number of compounds eluted and detected; B) separation (Rs) of the 2 critical pairs (epirubicin/doxorubicin, cyclophosphamide/ifosfamide); C) Number of co-eluted compounds; D) average asymmetry factors of all detected compounds; and E) retention time of the last compound to elute. Mobile phase: A: CO_2; B: MeOH + 2 % H_2O+ 20 mM NH_4HCO_2.

due to the presence of multiple fluorine atoms, while methotrexate functions as a Lewis base, owing to its numerous nitrogen atoms. Strong interactions may form between the two, preventing the elution of methotrexate.

The second criterion was the separation of two critical pairs among the target compounds which are essential to distinguish due to their similarity: epirubicin/doxorubicin (epimers) and cyclophosphamide/ifofamide (isomers). Resolution of these two pairs for each stationary phase can be found on Fig. 3. The first pair, epirubicin/doxorubicin, is the most challenging to separate because they are epimers, differing only by an axial-equatorial epimerization of a hydroxyl group at C4 in the amino sugar. Since their *m/z* transitions are not compound-specific, they share the same fragmentations, making it impossible to differentiate them via MS. Only three columns (DIOL, HILIC and C18) were able to separate this pair, with a resolution of 1.7, 1.7 and 2.1, respectively. The second pair, cyclophosphamide/ifosfamide, is easier to separate, due to their distinct molecular structure. However, because cyclophosphamide is one of the most frequently used CADs and is often prepared in high concentration in hospitals, co-elution with ifosfamide could result in ionization competition, with cyclophosphamide potentially dominating, which could compromise accurate quantification. These two molecules were resolved only on five columns (2-PIC, 1-AA, DEA, DIOL and HILIC) with resolutions ranging from 1.6 to 2.5.

The third criterion was the number of co-elutions. Minimizing co-elutions is essential to avoid ionization competition in the ESI source, which can result in signal loss for certain compounds and inaccurate quantitation. In trace analysis, signal suppression is one of the most significant challenges. 1-AA and C18 were the only columns with a score of 5, with respectively 10 and 11 co-eluted compounds. Other very good columns, scoring 3 or 4, were DIOL (14 co-eluted compounds), DEA and 2-PIC. The other columns exhibited more than 17 co-eluted compounds.

The fourth criterion was peak shapes, evaluated by measuring the asymmetry factor determined at 10 % of peak height. The only column, 1-AA, achieved a score of 5 and was considered excellent in terms of peak shape. In contrast, columns such as 2-EP and FP received scores of 1 or 2, indicating disappointing results. The remaining columns scored between 3 and 4, with the DIOL column notably receiving a score of 4, with an average asymmetry factor of 2.43. Examples of problematic compounds with distorted peak shape (pemetrexed, raltitrexed, topotecan, irinotecan) on the columns mentioned were illustrated in Fig. 4.

The fifth criterion was the retention time of the last compound, which helps optimizing overall analysis time. Actual proportions of organic modifier from the first to the last retention time, with consideration of the delay volume and dead volume, were reported on Fig. 5. Gradient delay volume was determined in a previous study [29] using the same SFC system and corresponded to 440 µL, while column dead volume of 438 µL was calculated with the column dimensions and its porosity of 0.62. The worst columns, scoring 1, were 2-PIC (last peak eluted at 10.88 min, with 92 % MeOH), 1-AA, FP and HILIC. In contrast, 2-EP (last peak eluted at 7.62 min, with 53 % MeOH) and BEH achieved the best results, with a score of 5. This result was unexpected, as 2-PIC and 2-EP are relatively chemically similar in terms of bonding, but show significant differences in retentivity for certain compounds. The DIOL column received a score of 3, with the last compound eluting at 9.55 min (75 % MeOH). No clear trend emerged between the retention time of the last peak and the number of co-eluted compounds. While some columns, such as 2-EP, BEH, DEA, DIOL, C18 and 1-AA, exhibited an inverse relationship between these parameters, other columns like HILIC, FP and 2-PIC show both high retention time for the last eluted compound and a high number of co-eluted compounds.

In the end, when considering all the parameters explored, the DIOL column emerged as the best compromise between the number of compounds successfully eluted, the separation of critical isomeric pairs, the number of co-eluting compounds, the average asymmetry factor of all compounds, and the retention time of the last eluted compound (Fig. 2). This stationary phase was therefore selected for further method development.

3.3. Evaluation of the nature and concentration of additives

The role of additives in SFC is to improve the chromatographic performance such as peak shape of the compounds being analyzed. This study evaluated the impact of the type and concentration of three different additives in combination with the modifier (MeOH): ammonium formate (20 and 50 mM), water (2 and 5 %) and NH_4F (either absent or at 1 mM). Both water and ammonium formate are commonly used in SFC to optimize peak shapes for ionizable compounds, such as those in this study. NH_4F is another interesting additive known for improving compound ionization in ESI and enhancing MS sensitivity in LC [30,31] and more recently in SFC [27]. In this work, seven different additives combinations were compared with a reference composed of 2 % water and 20 mM formic acid: A) 2 % H_2O, 20 mM ammonium formate, no NH_4F; B) 5 % H_2O, 20 mM ammonium formate, no NH_4F; C) 2 % H_2O, 50 mM ammonium formate, no NH_4F; D) 2 % H_2O, 20 mM ammonium formate, 1 mM NH_4F; E) 2 % H_2O, 50 mM ammonium formate, 1 mM NH_4F; F) 5 % H_2O, 50 mM ammonium formate, no NH_4F; and G) 5 % H_2O, 50 mM ammonium formate, 1 mM NH_4F. The peak shapes were visually assessed, and the mean asymmetry factor (measured at 10 % of the peak height) was calculated to evaluate the influence of each combination on chromatographic performance.

Among the 22 molecules studied, 15 compounds exhibit a similar peak shape across all tested additive combinations. In contrast, seven compounds (methotrexed, pemetrexed, raltitrexed, daunorubicin, idarubicin, topotecan and irinotecan) showed significant variations in peak shape depending on the type and concentration of the additives. Notably, these seven molecules, which were the most affected by the additives, were also among the most acidic or basic in the set. Methotrexate, pemetrexed, and raltitrexed are acidic, each containing two carboxylic acid groups with pK_a values around 4, while daunorubicin, idarubicin, topotecan, and irinotecan are basic, with amino groups having pK_a values between 9.1 and 11.0. The chromatograms of these seven compounds across the seven additive combinations tested are shown in Fig. 6. These analytes belong to three classes of CADs: camptothecin derivatives (irinotecan, topotecan), anthracyclines (daunorubicin, idarubicin), and folic acid antagonists (methotrexate, pemetrexed, raltitrexed). Therefore, the following interpretations of the additives effects will focus primarily on these seven compounds.

Some additives combinations were rapidly excluded from further evaluation for various reasons. All combinations containing 5 % water were discarded due to system overpressure during the reequilibrating step at only 2 %B. This is likely caused by phase separation (demixing) due to the very low solubility of water in a mobile phase with a very high proportion of supercritical CO_2 (only 0.1–0.3 % water depending on the pressure, temperature and amount of co-solvent). Additionally, experiments involving ammonium fluoride and differing by 20 mM or 50 mM ammonium formate, were rejected despite an improvement in average peak symmetry of 29 and 36 %, respectively. This was due to a significant reduction in MS signal intensity across all compounds, with an average reduction of 53 %. Sixteen compounds showed a signal loss greater than 50 %, with MTX showing the highest signal reduction at 89 %. Lastly, the impact of ammonium formate remains to be discussed. Using a higher concentration of ammonium formate proved to be the most effective way to improve peak shape of the seven compounds, increasing symmetry by 29 % compared to the reference, as illustrated in Fig. 6. This behavior could be explained by the elution of the invisible additive zones which can be more or less retained and provide peak deformation. When the solutes are eluting too close to the additive perturbation peak, a severe peak deformation can be observed due to co-elution. When the solutes are more retained than the additive, the deformation is caused by multilayer adsorption. The ammonium additive probably acts as a kosmotropic agent, promoting the multilayer adsorption of solutes to the stationary phase [32]. Thus, the optimal

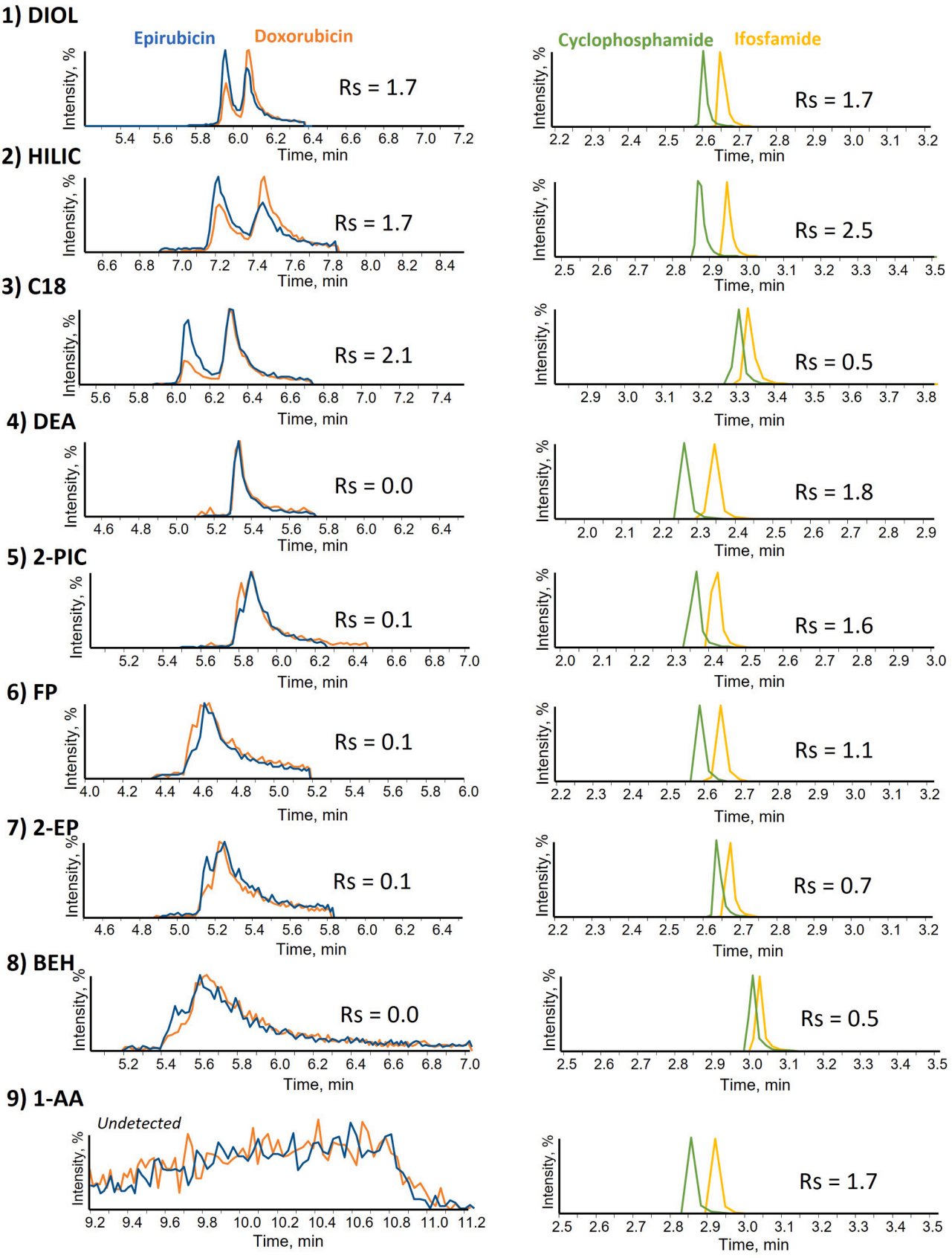

Fig. 3. Separation of two most critical peak pairs (left: epirubicin/doxorubicin; right: cyclophosphamide/ifosfamide) on nine different stationary phases. Mobile phase: A: CO_2; B: MeOH + 2 % H_2O+ 20 mM NH_4HCO_2. A sample containing a mixture of all analytes at 100 ng/mL was injected with a volume of 1 µL.

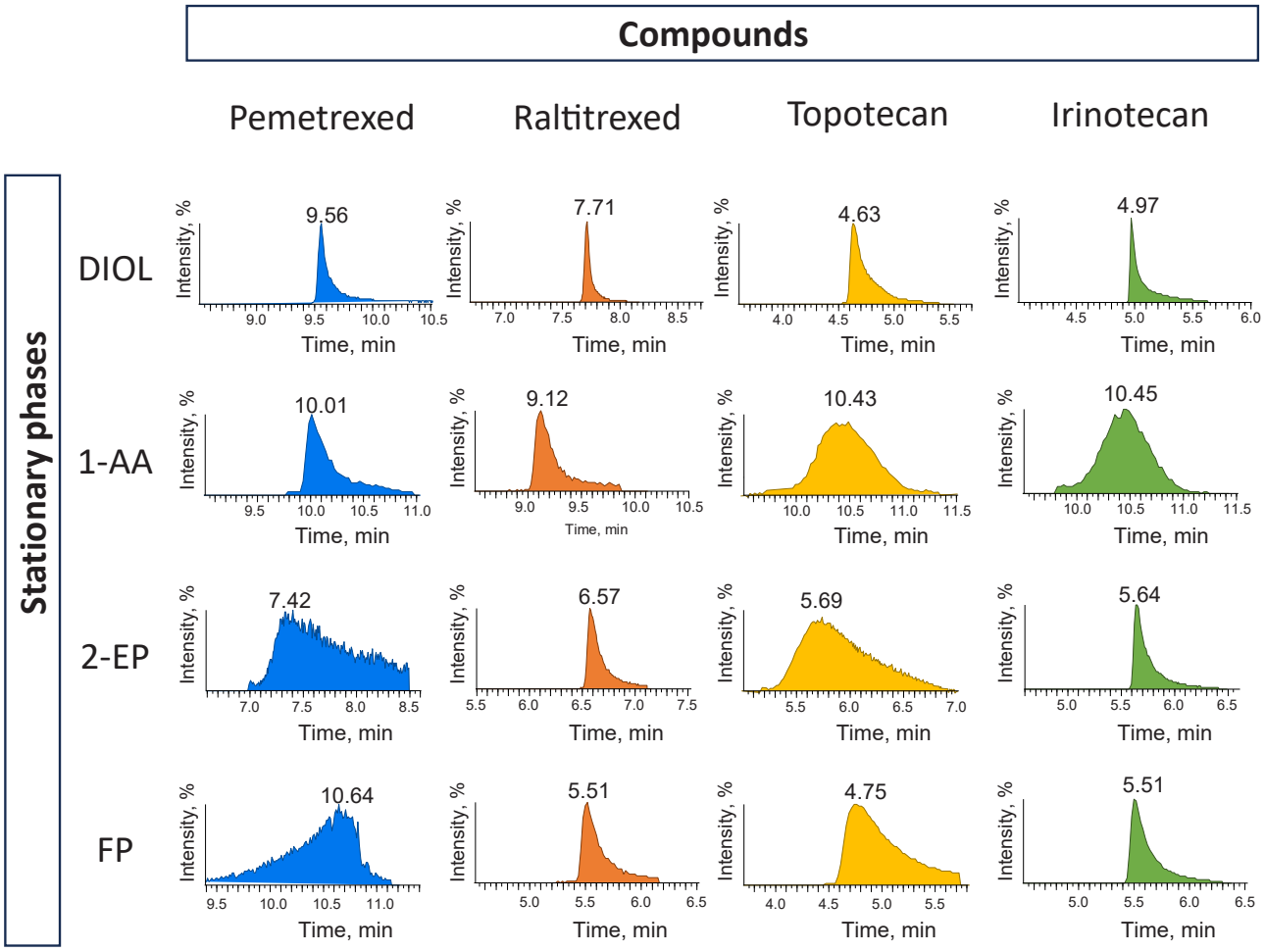

Fig. 4. Example of four compounds (pemetrexed, raltitrexed, topotecan, irinotecan) with distorted peak shape on various stationary phases. Mobile phase: A: CO_2; B: MeOH + 2 % H_2O+ 20 mM NH_4HCO_2. A sample containing a mixture of all analytes at 100 ng/mL was injected with a volume of 1 μL.

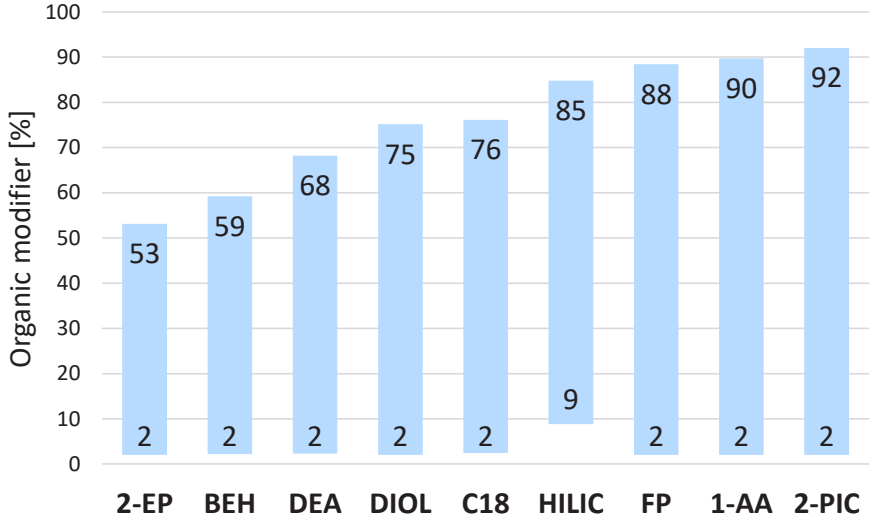

Fig. 5. Real organic modifier proportion from first to last retention on different stationary phases, considering delay volume and dead volume. Mobile phase: A: CO_2; B: MeOH + 2 % H_2O+ 20 mM NH_4HCO_2.

additive combination selected contains 2 % water and 50 mM ammonium formate in methanol.

3.4. Organic modifier composition

Different compositions of organic modifier can be used to adjust the polarity and eluent strength of the mobile phase, thereby affecting

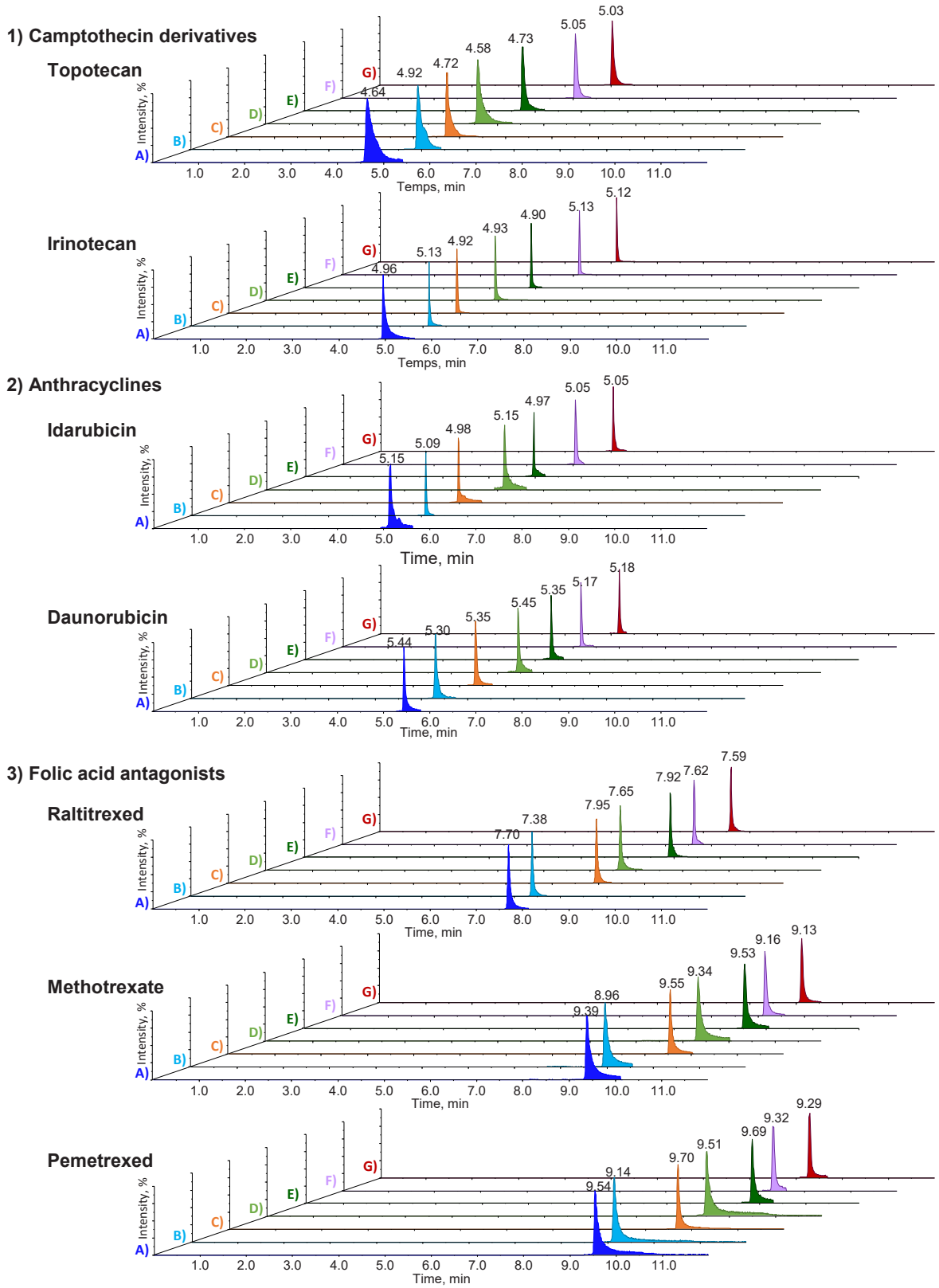

Fig. 6. Chromatograms of seven compounds (topotecan, irinotecan, idarubicin, daunorubicin, raltitrexed, methotrexed and pemetrexed) showing significant variations in peak shapes across the seven tested additives combinations. They belong to three anticancer families: 1) Camptothecin derivatives; 2) Anthracyclines; 3) Folic acid antagonists. The 7 additives combinations were as follows: A) 2 % H_2O, 20 mM NH_4HCO_2, no NH_4F (in dark blue); B) 5 % H_2O, 20 mM NH_4HCO_2, no NH_4F (in light blue); C) 2 % H_2O, 50 mM NH_4HCO_2, no NH_4F (in orange); D) 2 % H_2O, 20 mM NH_4HCO_2, 1 mM NH_4F (in light green); E) 2 % H_2O, 50 mM NH_4HCO_2, 1 mM NH_4F (in dark green); F) 5 % H_2O, 50 mM NH_4HCO_2, no NH_4F (in purple); G) 5 % H_2O, 50 mM NH_4HCO_2 (in red), 1 mM NH_4F. Column: Waters Torus DIOL. Organic modifier: MeOH. A sample containing a mixture of all analytes at 100 ng/mL was injected with a volume of 1 µL.

analyte retention and selectivity. In this study, the impact of organic modifier composition was investigated, by maintaining a base ratio of 80 % MeOH, while varying the remaining 20 % with other organic solvents (MeOH, EtOH, IPA and MeCN). All mobile phase also included 2 % water and 50 mM ammonium formate. The primary goal was to improve the separation of all compounds, especially the isomeric pairs (cyclophosphamide/ifosfamide and epirubicin/doxorubicin), using resolution as the comparison parameter. Peak symmetry was also assessed based on the average asymmetry factor at 10 % peak height for each compound.

Ternary mobile phases containing MeCN, EtOH or IPA systematically resulted in higher retention compared to pure MeOH, with the MeOH/MeCN mixture causing the most significant retention increase. This behavior was expected, since MeCN is an aprotic solvent with limited eluent strength in SFC. For alcohol-based modifiers, retention decreases as the alkyl chain length of the alcohol increases. This is again logical, since the hydrogen-bonding capacity follows the order: MeOH > EtOH > IPA, with MeOH having the strongest hydrogen-bonding capacity and IPA the weakest. This trend is attributed to the increasing steric hindrance from the bulkier alkyl groups in ethanol and isopropanol compared to methanol. Consequently, MeOH, being the most protic solvent, results in shorter retention times. Compared to pure MeOH, the maximum changes in retention times were 0.39 min with the MeOH/EtOH mixture (observed for raltitrexed and methotrexate), 0.74 min with the MeOH/IPA mixture (for raltitrexed), and 0.94 min with the MeOH/MeCN mixture (for raltitrexed).

Apart from retention, only slight changes in selectivity were noted when modifying the organic solvent. The elution order of the compounds remained consistent, except for a reversal in the elution order of dacarbazine and vincristine with the IPA mixture, and vincristine and paclitaxel with the MeCN mixture. Similar findings were reported by Galea et al., who also examined the impact of mobile phase composition on selectivity [33]. However, their experimental conditions differed, as they used higher proportion of the same three co-solvents, ranging from 50 % to 60 %, and their gradient range was narrower, limited to 5–40 %.

Fig. 7 shows the separation for the two most critical pairs under different mobile phase conditions, based on the modifier composition. The MeCN mixture slightly enhanced the resolution of the epirubicin/doxorubicin pair, while IPA improved resolution of the cyclophosphamide/ifosfamide pair. The addition for EtOH, however, did not offer any notable benefit in terms of resolution. Despite the selectivity changes observed with MeCN and IPA, the improvements were too limited to justify the use of a more complex ternary mobile phase for this analysis. In fact, when the resolution of one critical pair improved, the resolution of the other pair systematically decreased. Additionally, it is important to note that adding any of these three organic solvents to MeOH led to unpredictable system overpressure. No further experiments were conducted to investigate this issue. To ensure reproducibility and minimize

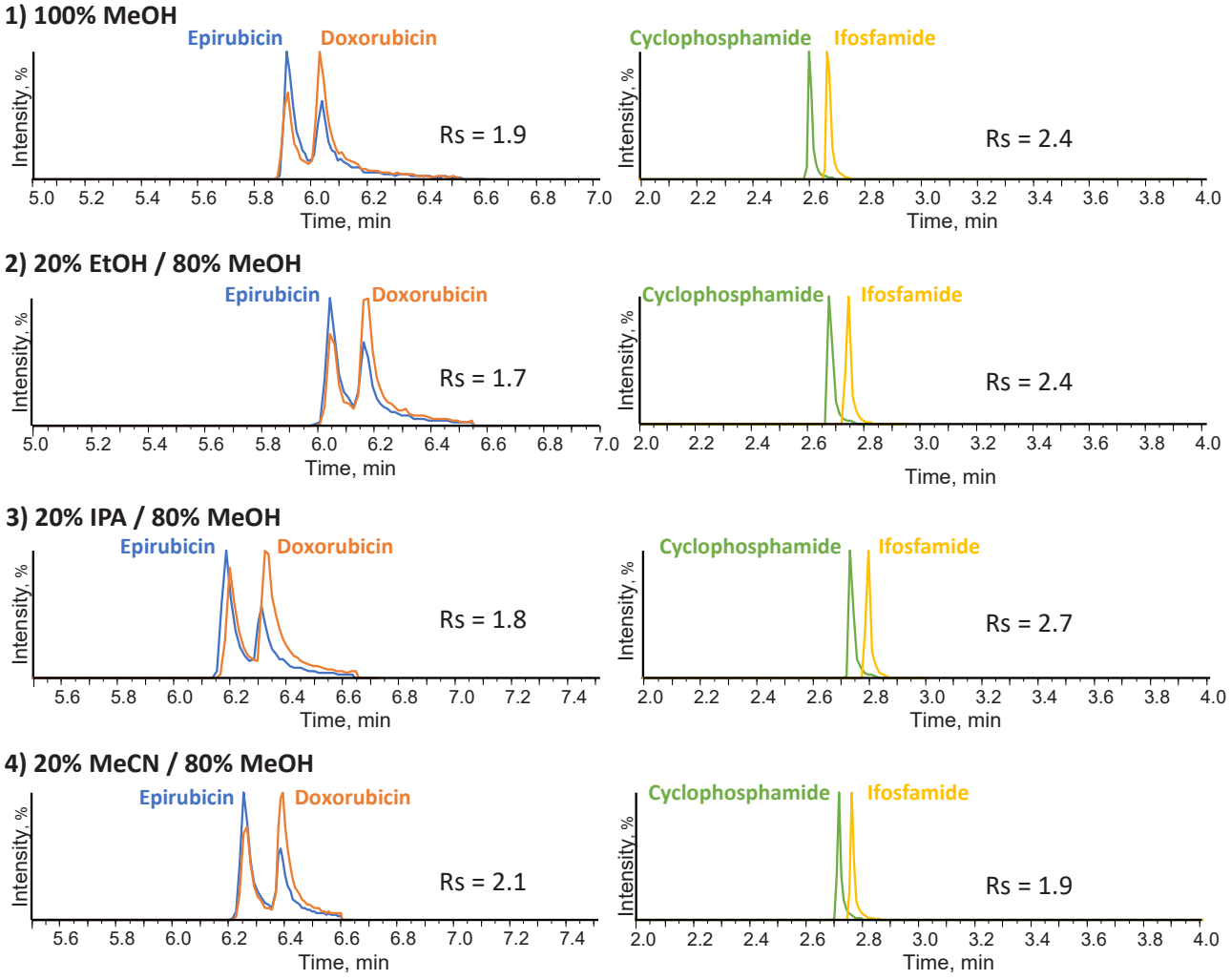

Fig. 7. Separation of the two most critical peak pairs (left: epirubicin/doxorubicin; right: cyclophosphamide/ifosfamide), based on the composition of organic modifier: 1) 100 % MeOH; 2) 20 % EtOH/80 % MeOH; 3) 20 % IPA/80 % MeOH; and 4) 20 % MeCN/80 % MeOH. Column: Waters Torus DIOL. Additives: 2 % H_2O + 50 mM NH_4HCO_2. A sample containing a mixture of all analytes at 100 ng/mL was injected with a volume of 1 µL.

risks associated, pure MeOH was chosen as the organic modifier.

3.5. Addition of other targets

To evaluate the applicability of this method for analyzing particularly challenging CADs, five additional compounds were included: mitomycin, azacitidine, cisplatin, oxaliplatin) and carboplatin. Individual standard solutions of each compounds were initially prepared in MeCN:H_2O (1:1) at two concentrations (100 and 400 ng/mL) for unambiguous identification. These compounds were then combined with the 22 other CADs into a single mixture to confirm the absence of any interferences. These five compounds were selected due to their frequent preparation in our hospital. Analyzing them by chromatographic methods can be challenging for various reasons. Platinum derivatives are known to form complexes, making it difficult to predict their behavior in supercritical fluids, although the low water content is advantageous. Additionally, cisplatin can exist in equilibrium with various complex forms in water, while oxaliplatin and carboplatin are prone to self-association in concentrated solutions. Azacitidine and mitomycin, on the other hand, are subject to hydrolysis in aqueous and acid solutions, respectively. For cisplatin, only its monohydrated form was observed, indicating that it was fully transformed under the applied conditions. This result was expected, as cisplatin is one of the most unstable CADs in aqueous conditions and small amount of water were still used in the developed method. Indeed, 2 % water was employed as an additive in the mobile phase and the mixture of standards were

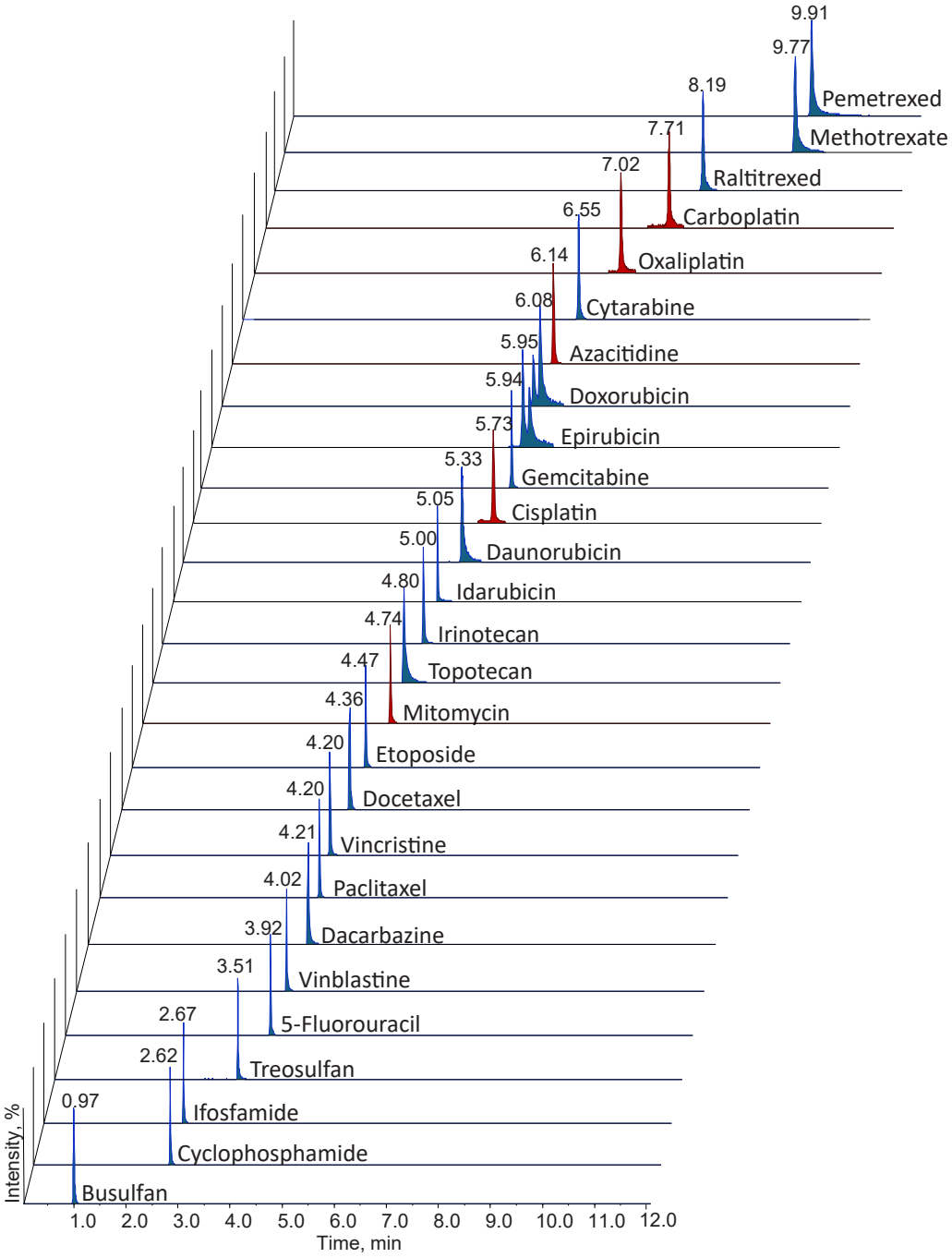

Fig. 8. Chromatogram of 22 initial (blue) and 5 additional (red) anticancer drugs evaluated. Columns: Waters Torus DIOL. Mobile phase: A: CO_2; B: MeOH with 2 % H_2O + 50 mM NH_4HCO_2. A sample containing a mixture of all analytes at 100 ng/mL in MeCN/H_2O (1:1) was injected with a volume of 1 µL.

prepared in water and acetonitrile (1:1). Even though the system contained a low proportion of water, the SFC method still managed to identify the monohydrate form of cisplatin, which was not achievable in previous method development in RPLC conducted in a water-rich environment. This developed method allowed the identification of cisplatin and the other additional CADs, however further experiments is required to refine the method and limit the presence of water, for example by testing other solvents for dilution.With the optimized chromatographic conditions, all five compounds were successfully identified alongside the initial set of 22 CADs, as shown in Fig. 8. Their retention time and asymmetry factor were summarized in Table 2. Regarding the co-elution, the three platinum derivatives (cisplatin, oxaliplatin and carboplatin) did not elute simultaneously with the other compounds and were detected at 5.73, 7.02 and 7.71, respectively. Mitomycin eluted closely with the next compound (topotecan) at 4.74 min, while azacitidine eluted near the previous one (doxorubicin) at 6.14 min. In terms of peak shape, all five additional CADs had an excellent asymmetry factor between 1.37 and 1.45.

The elution order can be associated with the family and structure of CADs. The first families to elute were alkylsuofnates (busulfan, treosulfan) and oxazophosphorines (cyclophosphamide, ifosfamide), which are small compounds with a molecular weight lower than 279 g/mol. Vinca alkaloids (vinblastine, vincristine) and taxanes (paclitaxel, docetaxel) eluted closely between 4.0 and 4.4 min, having in common the most complex and broadest structure with heterotetracylic groups. Camptothecin derivatives (topotecan, irinotecan) and anthracyclines (doxorubicin, epirubicin, daunorubicin, idarubicin), both having quite similar structures with their linear aromatic groups, eluted between 4.8 and 6.1 min. Pyrimidine antagonists with sugar linked to their base (gemcitabine, cytarabine, azacitidine), were all closely eluted around 5.9–6.6 min, except for 5-FU that is only composed of a nitrogenous base and eluted earlier at 3.92 min. Finally, the folic acid antagonists (i.e., raltitrexed, methotrexate, pemetrexed) were the most retained compounds, likely due to their ability to form strong hydrogen bonds with the diol column.

Table 2
Retention and asymmetry factor for the 27 compounds.

Analytes	Retention time [min]	Asymmetry factor
5-Fluorouracil	3.92	1.1
Azacitidine	4.14	0.9
Busulfan	1.01	1.2
Carboplatin	7.71	1.3
Cisplatin	5.73	1.4
Cyclophosphamide	2.62	1.3
Cytarabine	6.55	1.3
Dacarbazine	4.21	1.4
Daunorubicin	5.33	2.9
Docetaxel	4.36	1.1
Doxorubicin	6.08	0.9
Epirubicin	5.95	2.0
Etoposide	4.47	2.2
Gemcitabine	5.94	1.2
Idarubicin	5.05	1.3
Ifosfamide	2.67	0.9
Irinotecan	5.00	1.3
Methotrexate	9.77	3.3
Mitomycin	4.74	0.9
Oxaliplatin	7.02	1.0
Paclitaxel	4.20	1.1
Pemetrexed	9.91	2.5
Raltitrexed	8.19	2.3
Topotecan	4.80	1.4
Treosulfan	3.51	1.8
Vinblastine	4.02	1.2
Vincristine	4.20	1.2

3.6. Preliminary validation results

Before processing with a full validation of the developed analytical method, which constitutes Part II of this study, a series of analyses were conducted to have a first idea of the method's quantitative performance.

As a first step, the response functions of each compound were evaluated. Among the different tested models, the quadratic regression was selected because it provides the best determination coefficient with values higher than 0.993 in the concentration range between the LOQ and 200 ng/mL (Table 3). For all compounds, an internal standard was systematically selected. This selection was directed by the elution profile obtained under the optimized SFC conditions, and their commercial availability. Since 5-Fluorouracil was the only compound detected in the negative ESI mode, [^{13}C,$^{15}N_2$]-5-Fluorouracil was selected as internal standard for this particular drug. For the other target analytes detected in positive ESI mode, radiolabeled compounds such as [[$^{2}H_8$]-busulfan, [$^{2}H_8$]-cyclophosphamide, [$^{13}C_6$]-irinotecan, [^{13}C, $^{2}H_3$]-methotrexate, [$^{2}H_5$]-paclitaxel, [^{13}C, $^{15}N_2$]-5-fluorouracil, [$^{13}C_3$]-cytarabine) and [$^{2}H_{10}$]-oxaliplatin were chosen based on the physico-chemical properties and elution times of analytes.

Regarding the sensitivity of the method, Table 3 highlights two groups of compounds; those with an LOQ of 0.5 ng/mL and those with higher LOQs between 1 and 5 ng/mL. Compared to the LOQs determined by a reference LC-MS method [2] allowing the simultaneous analysis of only 22 CADs, similar sensitivities were obtained for 12 of the tested compounds (i.e. busulfan, cyclophosphamide, dacarbazine, docetaxel, doxorubicin, gemcitabine, idarubicin, ifosfamide, irinotecan, methotrexate, paclitaxel and topotecan). However, lower LOQ values (factor 10) were obtained for 5-fluorouracil, cytarabine, daunorubicin, etoposide, pemetrexed, raltitrexed, vincristine, vinblastine with the developed SFC-MS method. Only epirubicin gives a LOQ value 2 times higher under SFC conditions (10 ng/mL instead of 5 ng/mL). However, these values remain indicative because only the S/N was considered for their evaluation.

The concentration of six samples at 100 ng/mL was calculated from the response function obtained for each compound. Accuracy was expressed in percent as the ratio of the average measured concentration to the theoretical concentration. As reported in Table 4, accuracy values were comprised between 88 % and 117 %, with RSD values between 2 % and 21 %. Comparable results were previously reported with the LC-MS method [2].

3.7. Application of the SFC-MS method to wipe samples

To demonstrate the applicability of the SFC-MS method to real samples, the contamination rate of 27 CADs was measured on different places in hospital pharmacy chemotherapy compounding units. The sample locations included places in the logistic area (*e.g.* bench surfaces, storage of antineoplastic products, and equipment regularly handled by operators such as computer keyboards, mice and phones). In most samples, no trace of antineoplastic drugs was detected. Four samples with positive results, expressed in ng per sample, were analyzed by the developed SFC-MS as well as the LC-MS method [2] and are reported in Table 5. The use of different desorption solvents (water:acetonitrile for SFC and water for LC) could explain the slight difference in the obtained results. The presence of CADs on surfaces implies the implementation of corrective measures, such as a more efficient cleaning procedure with use of different cleaning solvents, or enhanced cleaning frequency to reduce the surface contamination.

Based on these results, the developed SFC-MS method appears to be a promising tool for the analysis of CADs in the hospital environment.

4. Conclusion

The primary objective of this study was to evaluate the applicability of a generic SFC-MS/MS method for the simultaneous qualitative

Table 3
Regression models for the 27 compounds.

Analytes	Internal standard	Concentration range [ng/mL]	Equation	Regression coefficient (R^2)
5-Fluorouracil	5-Fluorouracil-[^{13}C.$^{15}N_2$]	0.5–200	$y = 5.560e^{-3}x^2 + 1.33089x + 0.00737$	0.99778
Azacitidine	Cytarabin-[$^{13}C_3$]	0.5–200	$y = 2.219e^{-4}x^2 + 0.04643x + 0.00252$	0.99947
Busulfan	Busulfan-[$^{2}H_8$]	0.5–200	$y = 2.520e^{-3}x^2 + 0.93752x + 0.04222$	0.99783
Carboplatin	Cytarabin-[$^{13}C_3$]	5–200	$y = 2.054e^{-5}x^2 + 0.00402x + 0.00119$	0.99996
Cisplatin	Cytarabin-[$^{13}C_3$]	1–200	$y = 2.168e^{-4}x^2 + 0.04305x + 0.00732$	0.99920
Cyclophosphamide	Cyclophosphamide-[$^{2}H_8$]	0.5–200	$y = 1.248e^{-2}x^2 + 0.21538x + 0.13393$	0.99719
Cytarabine	Cytarabin-[$^{13}C_3$]	0.5–200	$y = 1.240e^{-3}x^2 + 0.66619x + 0.04032$	0.99913
Dacarbazine	Cytarabin-[$^{13}C_3$]	0.5–200	$y = 3.890e^{-3}x^2 + 1.69878x + 0.08050$	0.99908
Daunorubicin	Cytarabin-[$^{13}C_3$]	0.5–200	$y = 3.409e^{-4}x^2 + 0.02640x + 0.00330$	0.99952
Docetaxel	Paclitaxel-[$^{2}H_5$]	0.5–200	$y = 4.220e^{-3}x^2 + 0.84509x - 0.06158$	0.99963
Doxorubicin	Cytarabin-[$^{13}C_3$]	5–200	$y = 1.554e^{-4}x^2 + 0.01083x + 0.00083$	0.99984
Epirubicin	Cytarabin-[$^{13}C_3$]	10–200	$y = 9.234e^{-5}x^2 + 0.00839x - 0.00008$	0.99998
Etoposide	Cytarabin-[$^{13}C_3$]	0.5–200	$y = 1.280e^{-3}x^2 + 0.51186x + 0.01838$	0.99923
Gemcitabine	Cytarabin-[$^{13}C_3$]	0.5–200	$y = -1.890e^{-3}x^2 - 1.890e{-}3x + 0.05032$	0.99948
Idarubicin	Cytarabin-[$^{13}C_3$]	5–200	$y = 2.252e^{-4}x^2 + 0.01132x + 0.00601$	0.99310
Ifosfamide	Cyclophosphamide-[$^{2}H_8$]	0.5–200	$y = 2.430e^{-3}x^2 + 0.58363x + 0.03200$	0.99958
Irinotecan	Irinotecan-[$^{13}C_6$]	0.5–200	$y = 4.700e^{-3}x^2 + 0.72266x + 0.06506$	0.99804
Methotrexate	Methotrexate-[$^{13}C.^{2}H_3$]	0.5–200	$y = 2.380e^{-3}x^2 + 0.56248x + 0.06423$	0.99896
Mitomycin	Cytarabin-[$^{13}C_3$]	0.5–200	$y = 5.480e^{-3}x^2 + 1.25840x + 0.07986$	0.99782
Oxaliplatin	Oxaliplatin-[$^{2}H_{20}$]	5–200	$y = 9.810e^{-3}x^2 + 0.43905x + 0.06913$	0.99420
Paclitaxel	Paclitaxel-[$^{2}H_5$]	0.5–200	$y = -1.480e^{-3}x^2 + 0.99749x + 0.03549$	0.99742
Pemetrexed	Methotrexate-[$^{13}C.^{2}H_3$]	1–200	$y = 9.388e^{-5}x^2 + 0.03727x + 0.00454$	0.99966
Raltitrexed	Methotrexate-[$^{13}C.^{2}H_3$]	0.5–200	$y = 3.389e^{-4}x^2 + 0.32340x + 0.01671$	0.99842
Topotecan	Cytarabin-[$^{13}C_3$]	0.5–200	$y = 2.940e^{-3}x^2 + 0.32157x + 0.02385$	0.99945
Treosulfan	Cytarabin-[$^{13}C_3$]	0.5–200	$y = 7.808e^{-4}x^2 + 0.16620x + 0.00853$	0.99838
Vinblastine	Paclitaxel-[$^{2}H_5$]	0.5–200	$y = -4.007e^{-2}x^2 + 11.40275x - 1.25638$	0.99682
Vincristine	Paclitaxel-[$^{2}H_5$]	0.5–200	$y = 4.197e^{-1}x^2 + 4.53952x + 1.05201$	0.99611

Table 4
Accuracy for the 27 compounds. Results are based on six spiked samples at 100 ng/mL.

Analytes	Recovery (n = 6)		
	Mean	SD	RSD
5-Fluorouracil	88 %	7 %	8 %
Azacitidine	100 %	3 %	3 %
Busulfan	103 %	3 %	3 %
Carboplatin	96 %	7 %	7 %
Cisplatin	105 %	5 %	5 %
Cyclophosphamide	104 %	9 %	8 %
Cytarabine	103 %	3 %	3 %
Dacarbazine	98 %	2 %	2 %
Daunorubicin	103 %	10 %	9 %
Docetaxel	105 %	10 %	10 %
Doxorubicin	110 %	10 %	9 %
Epirubicin	99 %	13 %	13 %
Etoposide	101 %	5 %	5 %
Gemcitabine	107 %	5 %	5 %
Idarubicin	117 %	14 %	12 %
Ifosfamide	103 %	4 %	4 %
Irinotecan	104 %	11 %	11 %
Methotrexate	113 %	15 %	13 %
Mitomycin	106 %	4 %	4 %
Oxaliplatin	100 %	17 %	17 %
Paclitaxel	103 %	21 %	21 %
Pemetrexed	106 %	8 %	7 %
Raltitrexed	113 %	7 %	7 %
Topotecan	114 %	7 %	6 %
Treosulfan	109 %	4 %	4 %
Vinblastine	96 %	11 %	11 %
Vincristine	97 %	12 %	12 %

SD: standard deviation; RSD: relative standard deviation

Table 5
Calculated concentrations from three different surface samples analyzed using SFC-MS and LC-MS methods.

	SFC-MS method	LC-MS method
Unknown sample 1		
Irinotecan	4.4 [ng/sample]	3.3 [ng/sample]
Unknown sample 2		
Docetaxel	3.3 [ng/sample]	5.9 [ng/sample]
Unknown sample 3		
Treosulfan	3.9 [ng/sample]	5.4 [ng/sample]
Unknown sample 4		
Busulfan	460.6 [ng/sample]	439.7 [ng/sample]

analysis of several conventional anticancer drugs with a wide range of physico-chemical properties. Using a systematic approach consisting of testing different stationary phases and additives, a method was developed allowing the analysis of 22 anticancer molecules in less than 12 min. The use of a DIOL column (100 × 3.0 mm, 1.7 μm) with a gradient ranging from 2 % to 100 % organic modifier was employed to ensure adequate retention of the less retained compounds (as busulfan) and to elute the most polar ones (analogs of folic acid). To address the overpressure caused by the high organic solvent content, backpressure and flow rate were reduced in the second half of the gradient. The best separation was obtained with a mobile phase composed of 2 % H_2O and 50 mM ammonium formate in methanol. Furthermore, these optimized operating conditions appear promising for the analysis of CADs whose analysis in liquid chromatography is compromised by their instability in aqueous solution (azacitidine, mitomycin) and/or their unstable MS responses in the presence of water (cisplatin, oxaliplatin, carboplatin). Real samples were analyzed and compared with the LC-MS method. Both methods successfully identified the same traces of anticancer drugs, and the measured concentrations obtained from both methods showed no significant differences. Subsequent work aims to improve sensitivity, including the limit of detection and response function, and finally to validate the analytical method.

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CRediT authorship contribution statement

Fleury-Souverain Sandrine: Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Rudaz Serge:** Writing – review & editing, Resources, Project

administration. **Bonnabry Pascal:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Nguyen Nathalie:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Guillarme Davy:** Writing – review & editing, Writing – original draft, Resources, Methodology, Conceptualization.

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.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2025.116838.

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