Temperature-responsive comprehensive two-dimensional liquid chromatography coupled to high resolution mass spectrometry for the elucidation of the oxidative degradation processes of chemicals of environmental concern

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ABSTRACT

This study explores the possibilities offered by temperature-responsive liquid chromatography (TRLC) based comprehensive 2-dimensional liquid chromatography in combination with reversed-phase liquid chromatography (RPLC) for the analysis of degradation products formed upon oxidative treatment of persistent organic pollutants, in this case exemplified through carbamazepine (CBZ). The TRLC×RPLC combination offers the possibility to overcome peak overlap and incomplete separation encountered in 1D approaches, while the transfer of the purely aqueous mobile phase leads to refocusing of all analytes on the second dimension column. Consequently, this allows for about method-development free and hence, easier LC×LC. The study focuses on the oxidative degradation of CBZ, a compound of environmental concern due to its persistence in water bodies. The TRLC×RPLC combination effectively separates and identifies CBZ and its degradation products, while offering improved selectivity over the individual TRLC or RPLC separations. This allows gathering more understanding of the degradation cascade and allows real-time monitoring of the appearance and disappearance of various degradation products. The compatibility with high-resolution mass spectrometry is last shown, enabling identification of 21 CBZ-related products, nine of which were not previously reported in CBZ degradation studies. The approach's simplicity, optimization-free aspects, and ease of use make it a promising tool for the analysis of degradation pathways in environmental contaminants.

KEYWORDS: *analytical methods for advanced oxidation processes; wastewater; degradation products characterization; temperature-responsive liquid chromatography*

1. INTRODUCTION

Over the past decade, the increased adoption of commercial comprehensive two-dimensional liquid chromatography (LC×LC) instruments, coupled with a growing array of application methods, has enabled the enhanced physical separation of complex mixtures [1–3]. These achievements in chromatographic resolution were previously unattainable through conventional one-dimensional liquid chromatography with UV/MS approaches. Despite the increased efficiency obtainable with UHPLC compared to HPLC and the high resolving power of current mass spectrometry, in many cases 1D approaches provide inadequate or highly incomplete separation and easily confounded data interpretation [4,5]. This due to the occurrence of excessive peak overlap, especially when solutes are present over vastly differing concentration ranges [6]. In this way 2D-LC approaches also offer increasing potential for the analyses of organic contaminants of emerging environmental concern (CECs), and of their degradation products [2]. Such solutes represent a widening number of chemically disparate compounds which are released in the environment, while being either ecotoxic and/or posing a threat to human health [7]. Because often they are not or insufficiently removed via current water treatment processes various technologies are being developed to allow more efficient yet cost-affordable solutions. So-called Advanced Oxidation Processes (AOPs) provide therein promising prospects. Reactive species such as hydroxyl radicals ($^{\bullet}OH$), sulfate radicals ($SO_4^{\bullet-}$), superoxide radicals ($O_2^{\bullet-}$), and singlet oxygen ($^1O_2$) are produced in various ways to degrade organic pollutants partially or completely. The combination of UV and H_2O_2 is a straightforward yet very representative AOP approach to generate hydroxyl radicals [8,9]. Due to the poorly selective nature of radical reaction chemistry, the obtained degradation pathways are diverse and lead to many different and a priori unknown degradation products. The occurrence and disappearance of the latter is also rapidly evolving as function of reaction time and conditions [10]. Because the technological, energy and cost requirements to achieve complete mineralization of CECs are unrealistic when upscaling the approach to actual water treatment plants, it is important to understand which species can be formed in such processes [10,11]. Concomitantly it is necessary to have powerful analytical tools at one's disposal which allow rapid, simple, yet highly efficient separation and interpretation of the formed compounds [11]. Although LC×LC is particularly promising to address such challenges a current drawback of the technique is its relatively complex system configuration, whereby specialists' expertise is too often required to obtain useful data within acceptable time frames [12]. The main underlying challenge relies in overcoming the difficulty to transfer large plugs of first dimension (^{1}D) solvent to the second-dimension (^{2}D) column, without detrimentally affecting the second-dimension separation to such extent that eventually no useful data would be obtained any more. While a panoply of solutions has been indeed developed for this, such solutions usually come at the cost of simplicity, robustness, sensitivity or undermine the possibility for generic usage [13,14]. However, prior work by our group has demonstrated that the combination of purely aqueous separations in the first dimension with reversed-phase LC in the second dimension overcomes most of these issues because organic solutes present in the purely aqueous ^{1}D mobile phase inherently completely refocus at the head of the ^{2}D column [15]. This facilitates LC×LC method optimization, as this disconnects the ^{1}D and the ^{2}D method development. Hence both dimensions can be developed as one would do in conventional ^{1}D approaches, and it allows easy establishment of generic screening methods. However, only few purely aqueous separation modes are available allowing the analysis of mixtures of organic molecules, which comprise both neutral or charged compounds. Temperature-responsive liquid chromatography (TRLC), which relies on stimuli-responsive polymers covalently immobilized on the supporting material in a column (e.g., one aminopropyl silica), is interesting in this context. TRLC allows for the separation of organic molecules by using only water as mobile phase, whereby the retention of the compounds is controlled via temperature only [16,17]. This TRLC×RPLC approach proved already particularly effective for the enhanced, and method development free, separation of polyphenolic compounds in natural products, pharmaceutical mixtures or for chiral screening strategies [18–21]. Note that the potential of TRLC is inherently curbed by the the solubility limit of organic molecules in the mobile phase. However at typical analyte concentrations (ppb/ppm) as used in HPLC analyses, the

solubility limit of even highly apolar molecules is still high enough to allow analysis in purely aqeous TRLC [22]. When CECs are subjected to the mentioned oxidation processes a broad number of hydroxylated, carbonyl and other oxygen containing species are formed. Because this leads to compounds which are highly amendable to TRLC, in this work the suitability of the TRLC×RPLC-UV/HRMS is investigated for the separation of the oxidized solutes formed upon after oxidative treatment of environmentally problematic solutes. To the best of our knowledge, this approach represents the first demonstration of the potential of TRLC×RPLC in environmental analysis. Additionally, it marks the initial illustration of its compatibility with high-resolution mass spectrometry.

2. EXPERIMENTAL

2.1 Materials

Milli-Q grade water (18.2 mΩ) was purified and deionized in-house by a Milli-Q plus instrument from Millipore (Bedford, USA). Acetonitrile (ACN) was from ChemLab, Zedelgem, Belgium and formic acid (FA) 99 % LC-MS grade from VWR, Leuven, Belgium. Carbamazepine (5*H*-dibenze[b,f]azepine-5-carboxamide, CBZ) was supplied from Sigma-Aldrich, hydrogen peroxide wt. 35 % in water, stabilized was supplied by Acros (Geel, Belgium). CBZ stock solutions were prepared in ACN at a concentration of 1 mg mL^{-1}. The sample for forced degradation were obtained via dilution with water to 0.5 mg mL^{-1}. The samples were injected in the TRLC×RPLC system directly after degradation without alteration. Two identical columns (50 × 2.1 mm, silica particles 3 µm, 100 Å) coupled were used in the ^{1}D, packed with Poly(*N*,*N*-diethylacrylamide) (PDEAAm) modified silica, manufactured as described in Baert et al. [23] The polymer and column characteristics are outlined in the supporting information, S1.

2.2 Forced degradation

Carbamazepine was degraded using a UV lamp at a short wavelength (254 nm) with the addition of H_2O_2. The initial concentration of CBZ was 0.5 mg mL^{-1} in 50:50 ACN/H_2O, 10 % v/v hydrogen peroxide was added, and the sample was immediately placed under the UV lamp for a controlled period ranging from 10 to 60 minutes**.** Degradation parameters were experimentally selected, in order to reach CBZ degradation between 50 and 100 %. This to ensure the most informative insights in the degradation cascade.

2.3 Instrumentation

Measurements were performed on a 1290 Infinity II 2D-LC System controlled by the OpenLab CDS ChemStation Edition software (C.01.08[210], Agilent Technologies, Waldbronn, Germany). The instrument includes two binary pumps, a vial autosampler, a multicolumn thermostat, and a 2-position/8-port valve mounted to a valve drive interface equipped with two commercially available 80 µL loops. Detection was carried out with two diode array UV detectors containing a nano-flow cell in the ^{1}D (path length 10 mm, volume 1 µL) and a max-light cartridge cell in the ^{2}D (path length 60 mm, volume 1 µL). As a second detector in the ^{2}D, a Thermo Fisher Q Exactive Orbitrap MS (Thermo Fisher, Germany) was used. In the ^{2}D a InfinityLab Poroshell 120 EC-C18 column was used (3 × 50 mm, 1.9 µm, Agilent Technologies, Waldbronn, Germany). All the chromatograms and graphs were generated using PyCharm (JetBrains, python version 3.8, Czech Republic). The chemical structures and theoretical masses were obtained via ChemDraw (PerkinElmer, Seer Green, UK).

2.4 Chromatographic and MS conditions

^{1}D TRLC separations were performed with a water + 0.1% v/v FA mobile phase in the ^{1}D at a flow rate of 0.07 mL min^{-1} (see supporting information S2 for column performance). Injection volumes were 10 µL and the column temperatures in both dimensions were controlled at 45°C. The ^{2}D mobile phase was A) water + 0.1% v/v FA and B) ACN + 0.1% v/v FA in gradient elution mode from 2 to 55 % of B) in 0.75 minutes at a flow rate of 2 mL min^{-1}. The UV signal was

collected at wavelengths between 200 and 600 nm at 80 Hz, 254 nm is the wavelength plotted as it was showing the best response. The loops size was 80 µL, the modulation time 0.9 minutes with equilibration time of 0.15 min. After the UV detector the effluent was split 1:5 and directed respectively one part to the MS and the other to the waste. The MS analyses were carried out in full MS mode as well as MS/MS, positive polarity with a resolution of 70000, AGC target $1*10^{-6}$, maximum IT 30 ms and scan range 50 to 750 m/z. All the analyses were repeated in triplicate.

3. RESULTS AND DISCUSSION

CBZ was selected to study the effectiveness of TRLC×RPLC-UV/HRMS to separate, identify and monitor the formed degradation products. It is a compound of high environmental concern due to its persistence when subjected to conventional treatment processes, leading to its widespread occurrence in water bodies. Due to its semi-polar character (logP = 2.67) it is typically analyzed by reversed phase LC-MS on octadecyl silica (ODS) columns. A representative test sample for the method development was obtained by treating a 0.5 mg mL^{-1} CBZ solution for 1 hour with UV/H_2O_2. Subsequent analysis via 1D RPLC-MS allowed separation of some of the major formed products. However, as can be seen in Figure 1A the overlapping signals make the monitoring of the formed products challenging. Only the most prominent degradation products are well detectable and the co-elution of compounds complicates the ESI-MS detection of the less prevalent ones due to matrix effects. Note also that many molecular ions (shown in Table 1) of the hereby labeled degradation products could only be retroactively identified based on the actual information obtained by TRLC×RPLC-MS analyses (see further). Direct manual or automated treatment of the extraction ion chromatograms of ions of interest did not offer the possibility to detect and identify as many relevant ions. This mainly due to the complexity of the total (and base peak) chromatograms and the lack of separation in RPLC as consequence of the structural similarity between the degradation products (see Table S1). As a result, only a number of degradation products can be effectively followed in this way and clear interpretation of which solutes are formed at what stage of the process cannot be obtained. Similarly, analysis of the same sample by TRLC (Figure 1B) delivered an interesting selectivity, however, insufficient given the number of compounds expected, also hindered by extensive peak overlap due to the broader peak width and lack of retention for the very polar compounds.

Figure 1. A: LC-MS analysis of a degraded CBZ sample (60 min treatment) on C18 column. Representation of the reconstructed ion chromatogram based on the molecular ions of the degradation products shown in Table 1, which were effectively detected via 1D-RPLC-MS (conditions: water/ACN gradient +0.1% FA, flow rate 0.2 mL min^{-1}). B: Corresponding analysis by 1D-TRLC-MS (conditions: isocratic analysis with water +0.1% FA, flow rate 0.07 mL/min^{-1}). Methods reported in supporting information S3.

Despite the fact that the separations by RPLC and TRLC independently only allow for a partially satisfactory detection of the formed products, the combination of both modes in LC×LC offers promise. This because it combines different LC selectivities with a very effective type of LC×LC modulation, while offering the higher peak capacities typical of LC×LC. Consequently, a 2.1 mm I.D. ^{1}D TRLC column was combined with a 3 mm I.D. ^{2}D RPLC column, operated at 70 and 2000 µL min^{-1}, respectively. Loops of 80 µL volume were used and a modulation time of 0.9 min. This ensured for a maximal loop filling of 80% (to avoid losses due to the parabolic flow profiles [24]). TRLC was performed isothermally at 45°C to allow for the more retaining dehydrated state of the polymer, leading to enhanced retention and separation of the degradation products. The resulting contour plot shown in Figure 2A depicts a separation of the degradation products, with chromatographic resolutions with are not achievable on any of the individual dimensions. The coupling to HRMS also allows for easier compound identification due to the now achieved physical separation of many of the products. Additionally, UV/DAD detection now allows the easier profiling of the peak areas as a function of the degradation time.

Retention times values for both dimensions with relevant statistics of the triplicate measurements are reported in Table S2. It can be observed that the limited scan speed of the used HRMS instrumentation is causing peak broadening in the contour plots. While detrimental, this allowed peak identification (with high resolution MS data), whereby the UV data is then favored for the semi-quantitative (kinetic-monitoring) work as discussed below.

Figure 2. A: TRLC×RPLC analysis of carbamazepine degraded for 1 hour under UV/H_2O_2. B: corresponding HRMS based contour plot.

Figure 2 also shows that only the top and left half of the contour plot are effectively utilized. This is due to the hydrophobic retention which plays a major (yet not only) role in both RPLC and TRLC. However, a reasonable surface coverage of 55% and a peak capacity of 399 are obtained (calculations in supporting information S5). While much higher figures of merits have been reported for LC×LC, the optimization-free aspects of the current approach together with the ease of use, offer an elegant fit-for-purpose solution for the analysis of the degradation pathways of organic chemicals of environment concern such as CBZ. Nevertheless, as the oxidative processes proceeds, increasingly smaller and more polar solutes are formed. The latter become less retained and less separated by both TRLC and RPLC. This make the tool more amenable for the interpretation of the earlier stage generation of products based on the parent CBZ, which are anyhow the most interesting due to their structural similarity to the parent molecule. Because the high persistence of CBZ is for a large part related to the stability of the aromatic moieties, also the degradation products containing aromatic (and hence retained by RPLC an TRLC) groups are of most concern from an environmental point of view. HILIC based 1D and 2D methods would be required for the analysis of the most polar species. Also note that the implementation of shifted gradients did offer a somewhat enhanced surface coverage, but detrimentally affected the quality of the separations, especially for the more polar solutes eluting early in both ^{1}D and ^{2}D (Figure S5). The absolute masses allowed elucidation of the elemental compositions of the products. Combination with their fragmentation patterns, expected fragmentation pathways, partial information from literature and chromatographic retention, allowed for the allocation of 21 CBZ related products, 6 of which were not previously reported in earlier CBZ degradation studies (Table 1). Note that some more peaks are visible in the UV chromatogram. These were not included in the numbering as they could not be unambiguously assigned by mass spectrometry.

Table 1. Chromatographic and MS data for the 21 degradation products and carbamazepine structure. The structures related to peaks marked with * and the fragments marked with * were also found in other studies. Tentative structures proposed are in Table S4.

The chemical formulas in Table 1 indicate that unsurprisingly all the degradation products are more polar compared to CBZ. This is also translated into a decreasing RPLC retention. The aspect that some oxidation products of CBZ are also more retained by TRLC confirms earlier observations whereby also enhanced retention for phenolic, esterified, or other types of oxygenated aromatic structures are obtained compared to aromatic compounds as such. The table, figure 2A and the corresponding mass spectra in the supporting information (S8) show that various isomeric compounds are formed early on in the process. Unsurprisingly, direct hydroxylation of CBZ at various positions is one of the most prevalent processes (mono-hydroxylation is leading to compounds 10, 18, 19, 21, 22). Note that the exact location of the hydroxylation cannot be established with HRMS only. Double (compounds 9 and 20) and triple hydroxylation were also observed (compound 15). Other formed products involved the combination of the loss of functionalities (often the amide group) with oxygenations of the backbone structure. A detailed explanation of the degradation processes is provided in the supporting information (S11).

The developed approach offers the possibility to gather insight into the formation processes of the products. In Figure 3 the analysis of the of CBZ degraded for 10, 20 30, 40, 50 and 60 min is shown. The degradation of carbamazepine resulted

in the elimination of 66.5% of the compound in 60 min (Figure S7). Under the conditions used e.g., after 50 minutes many of the primary degradation products decrease in prevalence, while other processes take over (kinetics plots in supporting information S10). The approach is informative in the way that it allows visual observation of which processes are predominant, and in this way allows the design of enhanced degradation conditions.

Figure 3. TRLC×RPLC chromatograms of CBZ analyzed every 10 minutes during the degradation with UV/H_2O_2.

4. CONCLUSIONS

The potential of TRLC×RPLC-UV/MS is illustrated for the analysis of the degradation products formed during the oxidative treatment of a persistent organic pollutants such as carbamazepine. The approach proves well compatible with the oxidative treatment of pollutants strategy, whereby oxidized solutes species are formed which elute earlier in RPLC while an interesting isomeric selectivity appears possible by TRLC. In this way a distinction can be made both chromatographically and via MS of the single and multiply hydroxylated compounds in carbamazepine. Better monitoring of many degradation products becomes possible both by UV and MS detection. In general, the method allows rather straightforward elucidation of the type of degradation products formed and when they appear in the process.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the website.

Temperature-responsive LC×LC-MS for the elucidation of the oxidative degradation processes of chemicals of environmental concern (PDF).

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Peak	t_R ^{1}D (min)	t_R ^{2}D (s)	Measured mass (MH^+)	Exact mass (MH^+)	Δppm	Predicted formula	Reported in literature	Fragment peaks (m/z)	Isotopic pattern coverage (%)[a]	Change compared to CBZ
1	3.6	26.5	162.1487	162.1489	1.23	$C_8H_{20}O_2N$		144, 116	91.87	+7H, +O, -7C, -N
2	3.6	27.8	264.0865	264.0866	0.38	$C_{13}H_{14}O_5N$		246, 200	100.00	+4O, +H, -2C, -N
3*	4.5	29.2	180.0806	180.0808	1.11	$C_{13}H_{10}N$	[25–31]	152*	99.68	-$CONH_2$, -CH
4*	4.5	34.2	196.0756	196.0757	0.51	$C_{13}H_{10}ON$	[26,28,30–32]	168	99.68	-2C, -3H, -N
5	6.3	20.5	283.0712	283.0713	0.35	$C_{15}H_{11}O_4N_2$		265, 255, 240, 237, 212, 196, 184	98.46	+3O, -2H,
6*	6.3	24.2	287.1023	287.1026	1.04	$C_{15}H_{15}O_4N_2$	[28,29,33]	269*, 226, 208*, 180*	99.86	+3O, +2H,
7	8.1	26.4	254.0811	254.0812	0.39	$C_{15}H_{12}O_3N$		236, 210, 208, 180	100.00	+2O, -H, -N
8*	9.0	31.0	287.1023	287.1026	1.04	$C_{15}H_{15}O_4N_2$	[28,29,33]	269*, 226, 208*, 198, 180*	100.00	+3O, +2H
9*	9.0	37.3	269.0920	269.0921	0.37	$C_{15}H_{13}O_3N_2$	[31,32]	251, 226, 208, 180	99.27	+2O
10*	13.5	30.5	253.0970	253.0972	0.79	$C_{15}H_{13}O_2N_2$	[25,27–29,32,34]	236, 210, 208, 180*	100.00	+O
11*	11.7	35.0	208.0756	208.0757	0.48	$C_{14}H_{10}ON$	[32]	180	99.68	-C, -3H, -N
12	11.7	37.3	224.0703	224.0706	1.34	$C_{14}H_{10}O_2N$		206, 196	98.71	+O, -C, -3H, -N

13*	14.4	37.3	251.0813	251.0815	0.80	$C_{15}H_{11}O_2N_2$	[25,31]	251, 223*, 210, 208, 180	100.00	+O, -2H
14*	17.1	40.0	251.0813	251.0815	0.80	$C_{15}H_{11}O_2N_2$	[25,31]	251, 236, 223, 210, 208, 180	99.37	+O, -2H
15	13.5	33.4	285.0865	285.0870	1.75	$C_{15}H_{13}O_4N_2$		268, 242, 240, 224, 212, 196	98.60	+3O
16*	15.3	30.2	267.0761	267.0764	1.12	$C_{15}H_{11}O_3N_2$	[32]	239, 224, 196	99.90	-2H
17* (CBZ)	33.3	45.6	237.1019	237.1022	1.27	$C_{15}H_{13}ON_2$	[25–32]	220, 194, 192	99.27	
18*	32.4	41.3	253.0967	253.0972	1.98	$C_{15}H_{13}O_2N_2$	[9,26,27,32]	210*	100.00	+O
19*	32.4	41.3	253.0967	253.0972	1.98	$C_{15}H_{13}O_2N_2$	[29,31,32]	251, 236, 210, 208, 180	100.00	+O
20*	33.3	32.6	269.0917	269.0921	1.49	$C_{15}H_{13}O_2N_2$	[31,32]	226	99.27	+2O
21*	40.5	37.4	253.0964	253.0972	3.16	$C_{15}H_{13}O_2N_2$	[29,31,32]	210*, 208	100.00	+O
22*	32.4	41.3	253.0968	253.0972	1.58	$C_{15}H_{13}O_2N_2$	[9,26,27,32]	210*, 208	100.00	+O

[a] Mass error in ppm is calculated as (theoretical mass – exact mass) / theoretical mass * 10^6

[b] Pattern coverage is a measure of the summed intensity of the matching isotope peaks relative to the summed intensity of the theoretical isotope pattern.