Under the influence of light

New chromatographic tools for elucidating photodegradation mechanisms

den Uijl, M.J.

Publication date
2022

Citation for published version (APA):
7. Incorporating a liquid-core-waveguide cell in recycling liquid chromatography for detailed studies of photodegradation reactions

Abstract

In this work, a microfluidic photoreactor was embedded in a liquid-chromatography system. Mixtures were separated on an analytical column and compounds of interest were subsequently introduced into the light-reactor cell. After degradation, the content of the light-reactor cell was reinjected onto the same column, to separate the compound of interest from its degradation products. Because the separation was performed on the same column, degradation products could be linked to the components in the original mixture. The present system allowed further degradation cycles. A separated degradation product can be re-introduced into the photoreactor and irradiated again. The next generation of degradation products can again be separated on the same analytical column. This cycling procedure is an excellent tool to elucidate degradation pathways. This was demonstrated using riboflavin, better known as vitamin B2. By degrading it in the first cycle, its degradation products were isolated and subjected to a second degradation in the light-reactor cell. This allowed us to pinpoint secondary products and connect these with primary degradation products. The peak transfer between the first and second cycles and between the second and third cycles were optimized to provide recycling yields of 81% (± 2.3%) and 73% (± 5%), respectively, with the current setup.

Publication

Incorporating a liquid-core-waveguide cell in recycling liquid chromatography for detailed studies of photodegradation reactions

Mimi J. den Uijl, Ingrida Bagdonaite, Peter J. Schoenmakers, Bob W.J. Pirok, and Maarten R. van Bommel

J. Chromatogr. A, 2022, accepted with major revisions
7.1. Introduction

Many organic compounds can change under the influence of light. This process is called photodegradation. In food science, it is usually seen as undesirable, when healthy food ingredients, such as vitamins, degrade or when products lose their colour when stored over time [1–3]. In other fields, such as cultural heritage, photodegradation can lead to a loss of value, due to the fading of art objects, such as paintings and tapestries [4–6]. There are fields where photodegradation can be used advantageously, for example in advanced oxidation processes for water purification, where ultraviolet (UV) light is used in conjunction with hydrogen peroxide to reduce the organic-matter concentration [7,8].

Photodegradation, whether intentional or unwanted, is difficult to study. If a target molecule is degraded, many structurally related degradation products can be formed, which makes it hard to distinguish clear degradation pathways [9]. Moreover, degradation studies are often performed in simple setups, such as a solution in a beaker irradiated with a lamp. This requires a great deal of manual sample handling and makes it difficult to control factors, such as evaporation and the light dose [7,10]. Another disadvantage of simple setups is their inability to deal with complex matrices [11,12]. When food ingredients are degraded in pure-component aqueous solutions, degradation pathways may be different from those occurring in complex food or paint matrices [12,13]. Established methods to study photodegradation, such as the Microfading tester (MFT) and the Xenotest (XT) allow degradation of colorants applied directly on a solid matrix, while light-exposure rooms exist, for example to accelerate studies into the shelf lives of food products [14–16].

Recently, a new light-exposure cell was developed by our group, based on a liquid-core-waveguide (LCW) principle [17]. This light cell is made from Teflon-AF2400, a material with a lower refractive index than most liquids. In this way, the light can be captured within the cell and guided along its axis [18]. Photodegradation is performed in solution, making it compatible with liquid chromatography (LC). The degradation products formed in the LCW cell can be transferred to an on-line LC system, eliminating all manual sample handling [19]. Such photodegradation research can be completely automated by integrating the photoreactor with a multi-purpose sampler (MPS), which can inject samples into the cell and, after degradation through light irradiation, flush the degraded sample to the injection loop of the LC. The MPS can be programmed to start the LC run, clean the LCW cell, and prepare the system for another injection.

One problem that is not solved in any of the above techniques, is that very few compounds are available for study in their pure form. To circumvent this problem, an LC separation can
also be performed prior to degradation. To realize a system with separation stages both before and after photodegradation (i.e. LC-LCW-LC), an LCW cell was installed in a multiple-heart-cut two-dimensional LC system [20]. Using LC-LCW-LC, a sample could be separated on a first-dimension (1D) column and several fractions of the effluent could be stored in the multiple-heart-cut loops. These purified fractions (ideally pure-component peaks) were transferred to the LCW cell one by one and degraded, after which the reaction products were transferred to the second-dimension (2D) separation. This LC-LCW-LC system allowed studying the degradation of many (possibly unknown) compounds from a complex mixture in a light-exposure cell in an on-line, automated fashion for the first time. The setup was easy to use and yielded repeatable results. It was also the first time that photodegradation was employed as reaction modulation in two-dimensional LC (2D-LC) [21–23].

This LC-LCW-LC did have its limitations [20]. First of all, reversed-phase LC (RPLC) was used in both the 1D and 2D separations with similar mobile phases. The columns, however, were not identical, neither in terms of the stationary phase, nor in their dimensions. As a result, the 1D and 2D chromatograms were quite different, making it difficult to connect degradation products observed in the 2D chromatogram to impurities observed in the 1D chromatogram. Moreover, many of the compounds from a mixture selected for degradation yielded multiple degradation products, all of which inviting further study. The complex LC-LCW-LC setup did not allow performing such follow-up degradations on-line. Time profiles could be generated for each selected compound, but without further information it is nearly impossible to establish the complete network of structurally related degradation products. Lastly, although the LC-LCW-LC system was based on commercial 2D-LC hardware, it was complex and not straightforward to reproduce elsewhere [23,24]. Also, both the MPS-LCW-LC and LC-LCW-LC carry costs that prohibit their routine use in many laboratories.

One way to alleviate some of these issues is to use identical columns and conditions in the 1D and 2D separations. This would make it easier to track degradation products across chromatograms, but it does not remove the limitation of a single degradation step per compound. An elegant way to circumvent the latter problem is to redirect the effluent from the LCW cell back to the 1D column, i.e. LC followed by the LCW and back to the same LC, in short LC-(LCW-LC). This operation can be repeated to create an LC-(LCW-LC)$^n$ system that allows several ($n$) generations of photodegradation products to be studied for a single small sample in an automated fashion. Potentially, LC-(LCW-LC)$^n$ experiments allow highly detailed degradation studies, providing a path to improved understanding of degradation pathways. This new setup is experimentally much simpler than the LC-LCW-LC system, since no multiple-heart-cut valves and no 2D LC system (solvent-delivery system and column) are
needed. Also, the detector monitoring the 1D effluent can be eliminated from the setup. It is also considerably more simple and less expensive than an MPS-LCW-LC setup.

The objective of the present research is to create an automated on-line system that facilitates extensive photodegradation studies on small samples. We aim to generate information on the degradation of individual, potentially unknown components in complex matrices and to elucidate degradation pathways by tracing multiple generations of degradation products. To develop and demonstrate such a system we set out to study three samples, viz. an annatto extract (used as a food colorant), fuchsin (used as a textile dye), and vitamin B2.

7.2. Materials and Methods

7.2.1. Chemicals

Milli-Q water was obtained from a purification system (Arium 611UV, Sartorius, Goettingen, Germany; R=18.2 MΩ·cm). Methanol (MeOH, ULC/MS grade) was obtained from Biosolve (Valkenswaard, The Netherlands). Riboflavin (RF, ≥98%) was purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Acetic acid (≥95%) was purchased from Acros Organics (Geel, Belgium). All chemicals were used as purchased, except for RF which was used in a H₂O solution. Fuchsin was a gift from the Cultural Heritage Agency of The Netherlands (RCE, Amsterdam, The Netherlands). Annatto seeds were purchased from De Peperbol (Amsterdam, The Netherlands).

7.2.2. Instrumentation

7.2.2.1. Liquid Chromatography

All experiments were performed on an Agilent 1290 Infinity 2D-LC system (Agilent, Waldbronn, Germany) configured for one-dimensional operation. The system was comprised of a binary pump (G4220A), a diode-array detector (DAD, G4212A) equipped with an Agilent Max-Light Cartridge Cell (G4212-6008, V_{cell} = 1.0 μL), and an autosampler (G4226A). A 2-position 8-port valve (G4236A) was used with 40-μL loops installed. A reversed-phase liquid chromatography (RPLC) Zorbax Eclipse Plus C18 column (Agilent) was used with dimensions of 150 × 2.1 mm ID, 3.5 μm particle size. An 1100 Agilent isocratic pump (G1310A) was used to transfer the sample from the loops to the LCW cell and eventually to the injection loop.

7.2.2.2. Liquid-Core-Waveguide Cell

The LCW cell (ID 800 μm, OD 1000 μm, length 120 mm, volume 60 μL, pressure limit 0.5 MPa) was placed in a light box created by DaVinci Laboratory Solutions (Rotterdam, The Netherlands), which has been developed and validated in previous research, as described...
Chapter 7

in [19,20,25] and shown in Appendix D-1. The light source for degradation was a cold-white (400-700 nm) LED lamp (MCWHF2, Thorlabs, Newton, NJ, United States), which was coupled to a light fibre cable (M113L01, Thorlabs) with a core diameter of 400 μm, a UV/Vis collimator from Avantes (COL-UV/VIS, Apeldoorn, The Netherlands) and a plano-convex lens (f=35 mm, Thorlabs, LA4052-ML) to couple the light from the source into the LCW cell. The LED lamp was controlled by a LED driver (LEDD1B, Thorlabs). The lightbox was also equipped with a 6-port switching valve (EUHA, Vici, Houston, TX) to transfer the sample from the light box to the 2D separation. The 6-port switching valve was equipped with a loop of 100 μL.

7.2.3. Methods

7.2.3.1. Analytical Methods

In this work, an LC-(LCW\(\mathcal{U}\)LC)\(^n\) setup is developed to perform photodegradation. The binary pump, autosampler, 6-port injection valve, column, DAD, and 8-port valve described in the previous sections were connected in series. This latter valve was equipped with two 40 μL loops. An isocratic pump was used to transfer the 1D fractions stored in the loops to the LCW cell. This latter cell was coupled to the 6-port valve, equipped with a 100 μL loop, which was connected in between the autosampler and the column. A schematic overview of the setup is shown in Fig. 7.1.

For the LC analysis of all samples, mobile-phase components A and B consisted of mixtures of acidified water and MeOH in ratios of 99/1 [v/v] for mobile phase A and 1/99 [v/v] for mobile phase B. Mobile-phase component A contained 0.05% acetic acid (by volume). The flow rate of the binary pump was set to 0.4 mL/min. The gradient program started isocratically at 100% A from 0 min to 1 min, followed by a linear gradient to 100% B in 9 min, maintained for 1 min at 100% B, and finally returned to 100% A in 0.01 min. The composition was kept at 100% A for the remainder of time before the degraded mixture was re-injected onto the column. When the degraded sample was injected again, the same gradient program was started. The isocratic pump delivered a flow rate of 0.05 mL/min of mobile phase A when in operation. When the compound of interest eluted from the column, the 8-port valve was switched, and the compound was transferred to the LCW cell. After degradation it was transferred to the injection loop, after which it was re-injected onto the column. The specific time schedules are supplied in Appendix E-1. Depending on the application, this cycle was repeated either once or twice i.e. LC-(LCW\(\mathcal{U}\)LC)\(^n\) with \(n = 1\) or \(2\). The injection volume in all experiments was 5 μL.
7.2.3.2. Sample Preparation
A test mixture of riboflavin (RF, 100 mg/L) in H$_2$O was used. Fuchsin was dissolved in H$_2$O at a concentration of 100 mg/L.

Five annatto seeds (0.1620 grams) were extracted in 5 mL of MeOH/H$_2$O (75/25%) and sonicated for 20 min in an ultrasonic bath. After sonicating, the solution was passed through a PTFE filter (0.45 µm).

7.2.4. Data processing
The chromatograms were processed with Agilent OpenLAB CDS (Agilent, Santa Clara, CA, USA) software. Calculations were performed and figures were created with MATLAB R2020a (Mathworks, Woodshole, MA, USA) and Microsoft Excel.

7.3. Results & Discussion
In this research an online setup was developed to establish firm connections between sample components and their degradation products and to elucidate photodegradation mechanisms by repeated degradation of specific components. This system was built around a liquid-core-waveguide (LCW) cell, which was embedded in an LC setup with an extra 8-port valve and isocratic pump. The system is displayed in Fig. 7.1. One of the loops in the 8-port valve is coupled to the separating dimension, while the other is coupled to the degradation dimension.

**Figure 7.1.** Schematic setup of the cyclic separation setup. Blue and yellow indicate the two flow paths, i.e. blue is the separation dimension and yellow is the degradation dimension. $\text{gradP}$ is the gradient pump, INJ the injector, DAD the diode-array detector, $\text{isoP}$ the isocratic pump, LCW the liquid-core-waveguide cell, and W and W’ the waste bins. The left valve is indicated as recycling valve and the right valve as selection valve.
To test the transfer of a peak from the first separation to the next cycle, riboflavin was injected. The valve-switching times were optimized to facilitate efficient peak transfer. The time schedule of the method is provided in Appendix E-1. The fraction of the riboflavin peak transferred was determined with the light in the LCW cell off. It was found to be 81% (± 2.3%) and 73% (± 5%) between the first and the second separation, and between the second and third separation, respectively. A possible explanation of the difference in peak transfer and repeatability between the first and second transfer could be the slight shift in retention time in the second separation, because of the high injection volume. The fact that the sample transfer is not approaching 100% is probably due to the volume of the sample-transfer loops (40 µL), which is slightly smaller than the peak volume established using the DAD. Moreover, since these loops are placed behind the DAD in the streamline, the peak has probably experienced additional band broadening before it arrives at the loop, resulting in incomplete sample transfer. This effect is much smaller in the recycling valve, since the loop used for re-injection is much larger than the LCW cell (100 µL and 60 µL, respectively). Nevertheless, the experiments clearly showed that we can transfer the vast majority of a selected component to the LCW cell and subsequently reinject the degraded fraction in the column. In future work, the 40-µL loops may be larger to increase the sample transfer. Finally, it should be noted that in the present set up, the injection loop of 100 µL was not inserted in the flow line during the initial injection. This resulted in a peak shift of 0.25 min for compounds in the second or third separation. This can be remedied by modifying the setup such that the loop is inserted in the flow stream also during the initial injection.

7.3.1. Relating degraded fraction to initial separation

To demonstrate the versatility of the new setup we studied the degradation of fuchsin. Fuchsin is a synthetic organic colorant that was one of the most-important dyes for textile colouring in the 19th and 20th centuries [26–29]. Later it was applied in food, for example in wine and in sausages [26]. In more recent years, it has been used as biological staining agent and as a model compound for benchmarking waste-water-treatment facilities [30,31]. Fuchsin is a triarylmethane dye, with a methyl group at the meta position on one of the aromatic rings (see Appendix D-4). It is a starting material for the entire class of triarylmethane dyes. However, fuchsin is only available as a mixture of four compounds that differ in their degrees of methylation, i.e. not methylated (a, known as pararosaniline and as Magenta 0), singly methylated (b, known as fuchsin and as Magenta I), doubly methylated (c, known as Magenta II) and triply methylated (d, known as new fuchsin or Magenta III). The lower-case letters a-d refer to the initial chromatogram (blue line) in Fig. 7.2 (see Appendix D-4). While Magenta 0 and Magenta III are available as pure compounds, the other ones are not. Studying photodegradation of Magenta I or Magenta II is not possible in a mixture, since all four
components will likely yield comparable degradation products. This renders it impossible to link the degradation products to a specific parent molecule. Using the present setup, Magenta I was isolated from the fuchsin mixture and transferred to the light cell. It was either transferred through the light-exposure cell with the light off (purple line) or degraded for four hours (yellow line). The results of these experiments are shown in Fig. 7.2.

The figure shows the three separations overlayed. The fuchsin can be largely separated from all other peaks and reinjected in the system, without any other products in the chromatogram, as is evident from the purple trace in Fig. 7.2. In the degraded fraction (yellow trace) the original fuchsin peak is still visible, but some degradation products have formed. First of all, the demethylated product has been formed, which was already present in the first mixture (peak a). This confirms the hypothesis that one pathway in fuchsin photodegradation is the loss of methyl groups, leading to pararosaniline. Next to that, some very small peaks appear in the range from 8 min to 10 min (indicated by arrows). These small peaks are also present in the 1D chromatogram but have not been identified in this study. A next step in our work will be to couple the setup to a mass spectrometer to try and identify these products and to propose degradation pathways.
Next, a more-complex sample was analysed, *i.e.* an extract of annatto seeds. These seeds, originating from the achiote tree, are often used to produce a lipophilic natural food dye for products such as butter, cheese, and ice cream [32,33]. Its main compound is bixin, a carotenoid, with a yellow to orange colour. In Fig. 7.3, the chromatogram is shown of an extract of the annatto seeds (MeOH/H₂O, 75/25 [v/v]) at 450 nm. Bixin is clearly identified as the most lipophilic component in the mixture. The other (colouring) compounds found in the mixture elute before this main product. Since many of these components showed similar DAD spectra, we expect that they have structures similar to bixin with some slight modifications. When degrading annatto-seed extracts, degradation products cannot be distinguished from the minor components already present in the mixture. Therefore, bixin should be first isolated from the mixture. After isolation and reinjection (but without intermediate irradiation), the chromatogram consists only of bixin, meaning it was successfully isolated (see Fig. 7.3, purple trace). The chromatogram of the degraded fraction (irradiated for 2 h) is shown as the yellow trace in Fig. 7.3. Beside the bixin peak, which has clearly decreased in area, indicating degradation, some degradation products are seen in the chromatogram, the peaks of which correspond with those of components that were present in the starting mixture (see insert in Fig. 7.3). This suggests that bixin had already degraded to some extent prior to analysis.

**Figure 7.3.** Chromatograms of the initial annatto extract (blue trace), the reinjected fraction containing bixin without irradiation (purple trace) and after irradiation during 2 h (yellow trace). All chromatograms were recorded at 450 nm. Note that the time axis is corresponding to the initial separation. The other two chromatograms were aligned. Expansions of parts of the three chromatograms are plotted in the insert.
Caution should be taken in connecting degradation products with peaks present in the starting extract before confirmation experiments have been performed, for example with mass spectrometry. Moreover, the chromatograms shown were recorded at a wavelength of 450 nm, which implies that any products that do not absorb at this wavelength could not be observed.

7.3.2. Elucidation of degradation mechanisms

The current setup allows connecting degradation products to compounds in a mixture, as shown in section 7.3.1. It also makes it possible to study the degradation (and degradation mechanisms) of compounds that are not – or not easily – available in their pure form. This is a massive advantage for compounds that are prone to degradation. One example is riboflavin, known as vitamin B2, shown in Fig. 7.4A. Riboflavin is known to be very susceptible to light, which reduces its health effects in food or in supplements. When riboflavin is degraded, a mixture results that consists of (at least) seven different degradation products, as illustrated in Fig. 7.4B. The relation between these degradation products is hard to establish, since they could not be isolated previously.

Using the present setup both formyl-methyl flavin (FMF, Fig. 7.4C) and lumichrome (LCH, Fig. 7.4D) could be effectively isolated from the degradation mixture and subsequently subjected to a second degradation cycle with a similar dose and duration (30 min) as the initial degradation of the riboflavin mixture. In case of FMF it can be seen that a small amount of riboflavin is transferred to the second degradation cycle. This is probably due to the band broadening and incomplete chromatographic resolution (see also the beginning of Section 7.3). The degradation pattern looks similar to that in Fig. 7.4B, indicating that all these peaks may represent secondary degradation products of FMF. This is supported by literature [34,35],...
indicating that previously proposed degradation mechanisms can be verified using our developed system. Besides FMF, LCH was isolated and degraded yielding no new degradation products (Fig. 7.4D). This shows that LCH is much more stable under these conditions than RF. Since the absorption spectrum of LCH is also shifted to slightly lower wavelengths than that of RF, changing the irradiation source may result in more degradation of LCH.

7.4. Concluding remarks

In this paper, a novel setup was described featuring a liquid-core-waveguide light-exposure cell that allows online photodegradation. The fraction of the peak that was transferred was optimized to 81% (± 2.3%) and 73% (± 5%) between the first and the second separation, and the second and third separation, respectively. This system allows the user to readily connect degradation products to compounds present in the starting mixture. This was demonstrated with a degradation of fuchsin and of an annatto extract. In both these cases, the major degradation product was already found to be present at a significant level prior to degradation. The new setup also facilitates the elucidation of degradation mechanism, which was confirmed by performing applying it to riboflavin, a compound for which many photodegradation pathways had been proposed. The setup required fewer liquid-chromatography (LC) modules, making it less complex, cheaper, and more user-friendly than a previously proposed setup based on two-dimensional LC. The new system can operate with a basic injection device; it does not require a multi-purpose sampling system. The only additions to a standard LC instrument are the liquid-core-waveguide-cell box, an 8-port or 6-port valve, and a (low-pressure) isocratic pump.
References


