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# Observation and confirmation of oxidation reactions occurring on ultra-high-performance liquid chromatography columns

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**RATIONALE:** Ultra-high-performance liquid chromatography coupled with electrospray ionization mass spectrometry (UPLC/ESI-MS) has been frequently used for chemical analysis. A redox reaction in the ESI source has been observed during the ionization process. However, it is still unclear whether this redox reaction can take place on UPLC columns.
**METHODS:** In this study, the oxidation reactions potentially occurring on UPLC columns were investigated using polyphenols including baicalin, baicalein, propyl gallate (PG), quercetin-3-rhamnoside (QR), rutin, naringin and 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside (THS-G) as model compounds. The on-column oxidation reaction was ascertained by post-column infusion of antioxidants such as ammonium sulfide ($(NH_4)_2S$). The oxidized products were reduced to their parent forms in the ESI source. This on-column oxidation reaction was further confirmed by means of post-column infusion of baicalin solution.
**RESULTS:** On-column oxidation reactions were observed and confirmed for baicalin, baicalein, PG, rutin, and QR. The exact reaction site was located at the outlet frits of the UPLC columns. $(NH_4)_2S$ was proved to be the most suitable reducing agent among the tested antioxidants for eliminating negative effects caused by on-column oxidation reaction. It was subsequently proposed to be an efficient additive to suppress oxidation reactions in the ESI source.
**CONCLUSIONS:** Oxidation reactions can take place at the outlet frits of UPLC columns. Ascertaining on-column oxidation reactions and consequently eliminating relevant negative effects are of great interest for determination of oxidation-sensitive compounds such as polyphenols. 

Ultra-high-performance liquid chromatography coupled with mass spectrometry (UPLC/MS) has been widely used to determine a variety of compounds in various complex samples due to its capability of high throughput and exceptional sensitivity and selectivity.[1,2] However, this highly efficient and versatile instrument is not flawless. For instance, potential interference caused by phase II metabolites could impair the reliability of the analysis.[3] A much more serious issue concerning UPLC/MS and LC/MS was the redox reaction potentially occurring in the electrospray ionization (ESI) source,[4] which could damage analytes and consequently affect analytical results.[5,6] Previous studies have reported such redox reactions occurring in an ESI source.[7–10] It is easy to understand that compounds with relatively low redox potential can be oxidized in a positive ESI source and those with relatively high redox potential could be reduced in a negative ESI source.[11] Redox reactions taking place in the ESI source have been well studied in terms of mechanisms, theories, and applications.[12,13] For instance, the redox reaction in the ESI source was often used for the determination of haloquinones that could not be directly ionized by ESI-MS.[14–16] However, the redox reaction may take place not only in the ESI source, but also on UPLC columns.

Chemical reactions (e.g., degradation of analytes) occurring on LC columns have been reported by some studies concerning degradation and/or deconjugation of polymers, biomacromolecules, and covalently bonded colloidal assemblies on size-exclusion chromatography (SEC) columns.[17–22] In 1984, Barth and Carlin comprehensively reviewed the studies focusing on degradation of macromolecules on SEC columns.[23] Striegel *et al.* confirmed the degradation of the polysaccharide alternan on SEC columns.[18] Degradation of macromolecules on LC columns is very complex and has not been sufficiently studied yet. It has been found that the scission of macromolecule chains was caused by two kinds of forces, the shear force and the extensional force. Both forces can be produced in LC systems.[24] In the study of Uliyanchenkoa *et al.*, column frits were considered to be the main sources of the degradation of

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polymers on UPLC columns.[24] However, column frits were reported to make only a secondary contribution, or no contribution at all, to polymer degradation on LC columns when the frit radius is bigger than or equal to the hydraulic radius of the column.[25]

Chemical reactions of small-molecule analytes taking place on HPLC columns have been rarely reported so far. Dithiocarbamates were found to readily chelate with metal components on HPLC columns. This chelation was possibly in association with the stainless steel frits.[26,27] Dimerization and nitrosation of amines occurring on HPLC columns have been reported when using high-pH mobile phases containing ammonium hydroxide and acetonitrile (ACN) for separation.[28,29]

A redox (oxidation) reaction occurring on UPLC columns may negatively affect analysis of compounds that are susceptible to oxidation, such as polyphenolic compounds. Thus, it is necessary to ascertain the redox (oxidation) reaction occurring on UPLC columns and consequently find effective treatment to eliminate the negative effects. In addition, exploration of the exact site(s) and possible mechanisms of the oxidation reaction is also worthwhile. Nonetheless, redox reactions on UPLC columns have not been reported in literature to date.

Recently, we unintentionally observed an oxidation reaction during the instrumental analysis of polyphenolic compounds using UPLC/ESI-MS, and then suspected it might occur on the UPLC columns. Thereafter, a comprehensive study was performed to investigate whether the oxidation reaction could occur on UPLC columns. Seven polyphenols, including baicalin, baicalein, propyl gallate (PG), quercetin-3-rhamnoside (QR), rutin, naringin and 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside (THS-G), were chosen as model compounds. On-column oxidation was ascertained and confirmed through post-column infusion and quadrupole time-of-flight (QTOF) high-resolution mass spectrometry (HRMS). Precautions and possible solutions were further proposed to eliminate negative effects caused by on-column oxidation reactions.

## EXPERIMENTAL

### Chemicals and materials

Standards baicalin (purity ≥98%), baicalein (purity ≥98%), rutin (purity ≥98%), QR (purity ≥99%), naringin (purity ≥99%) and THS-G (purity ≥98%) were purchased from Nature-Standard Corporation (Shanghai, China). The structures and other information about these compounds are provided in Supplementary Table S1 (see Supporting Information). Antioxidants $(NH_4)_2S$ (S% ≥ 8%), ascorbic acid (VC, analytical reagent grade), formaldehyde (analytical reagent grade) were bought from Guangzhou Chemical Reagent Co., Ltd (Guangzhou, China). Hydroquinone (purity ≥99%), *o*-phenylenediamine (purity: 99.5%), resorcinol (purity: 99%), phloroglucinol (purity ≥99%), and formic acid (FA) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). PG (purity: 99%), catechol (purity: 98.5%) and dopamine hydrochloride (purity: 98.6%) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). HPLC grade methanol (MeOH) and acetonitrile (ACN) were purchased from Merck Corp. (Darmstadt, Germany). Ultrapure water (electrical resistivity: 18.2 MΩcm) was prepared with a water purification system from Millipore Corporation (Bellerica, MA, USA).

### Instrumentation

The UPLC/MS system consisted of an Acquity™ Waters UPLC system and a Synapt QTOF mass spectrometer (Waters Corp., Manchester, UK). The syringe pump was purchased from Harvard Apparatus (Harvard Pump 11 Plus, Holliston, MA, USA).

### Preparation of solutions

Each standard was accurately weighed and dissolved in MeOH to obtain a stock solution of 1.00 mg/mL. Working solutions of 1.00 μg/mL were further prepared by diluting the stock solutions with MeOH/$H_2O$ (1:1, v/v). All solutions were kept at 4°C in a refrigerator prior to use.

Each antioxidant was accurately weighed and dissolved in ultrapure water to obtain a target concentration of 10 mM, except $(NH_4)_2S$ of which the co cncentration was 1.7 mM. A baicalin solution (20.0 ng/mL) used for post-column infusion experiment was prepared by diluting the baicalin solution of 1.00 μg/mL with ultrapure water.

### UPLC/MS working conditions

Mobile phase A was ultra-pure water containing 0.1% FA, and mobile phase B was ACN. Separation was mainly performed on a Waters BEH C18 UPLC column (2.1 × 50 mm, 1.7 μm; Waters, Milford, MA, USA), a Waters HSS T3 column (2.1 × 100 mm, 1.8 μm) and a Waters CSH C18 UPLC column (2.1 × 100 mm, 1.7 μm). Other UPLC columns such as a Waters CSH C18 column (2.1 × 50 mm, 1.7 μm), a Waters BEH C18 column (2.1 × 150 mm, 1.7 μm) and some HPLC columns including a Waters XBridge™ C8 column (4.6 × 250 mm, 5 μm), a Diamonsil® C18(2) column (4.6 × 250 mm, 5 μm; Dikma, Beijing, China), and an Ultimate® AQ-C18 column (4.6 × 150 mm, 3 μm; Welch Materials, Shanghai, China) were also tested. Gradient elution programs are documented in Supplementary Tables S2 and S3 (see Supporting Information). The temperature of the column oven was maintained at 30°C and the injection volume was 5 μL.

An ESI source operated in both positive and negative modes was applied. The working conditions of the ion source are documented in Supplementary Table S4 (see Supporting Information). The resolution of the mass spectrometer was calibrated in real time with leucine enkephalin (L-ENK) (*m/z* 556.2771) at 200 ng/mL. The instrument control and data processing were performed with MassLynx V4.1.

### Post-column infusion experiment

Post-column infusion was performed with a Harvard syringe pump. At first, the infusion solution (antioxidant or model compound) was infused into the three-way pipe (T-pipe), followed by combination with the post-column mobile phase, and then was directed to the ESI source. Secondly, a solution containing a model compound or a compound mixture was injected onto the UPLC/MS system using an autosampler. The flow rates of the post-column infusion of the reducing

agent solutions varied upon requirements. A baicalin solution (20 ng/mL) was used for the post-column infusion at a flow rate of 10 μL/min.

### UPLC+UPLC experiment

Two UPLC columns [Waters BEH C18 column (2.1 × 50 mm, 1.7 μm) and Waters HSS T3 (2.1 × 100 mm, 1.8 μm) connected in serial (UPLC+UPLC) were employed to investigate separation of the model compounds and the oxidation products. An isocratic elution program with ACN/$H_2O$ (40:60, v/v) at a flow rate of 300 μL/min was used to enhance the separation.

## RESULTS AND DISCUSSION

### General outline

The workflow and general findings of this study are illustrated in Supplementary Fig. S1 (see Supporting Information) and Fig. 1, respectively. A polyphenolic compound susceptible to oxidation would undergo ultra-high pressure and intensive surface effect caused by column packing particles with diameter size of sub-2 micron when it was injected onto a UPLC system. It was speculated that the surface effect of the packing particles and the activated metals in a UPLC column catalyzed an on-column oxidation reaction, promoting the reaction between the polyphenolic compound and the dissolved oxygen or other possible oxidizing agents in the mobile phases. For all the model polyphenolic compounds, their possible oxidization products possess an *ortho*-diquinone group in their structures (Table 1).

### Observation of abnormal ions

The abnormal ions of baicalin ($[M–H]^+$/$[M–3H]^-$) with loss of two hydrogen atoms relative to the corresponding normal precursor ions ($[M+H]^+$/$[M–H]^-$) were unambiguously found with ESI-MS, having relatively high signal intensity in comparison with the corresponding normal precursor ions in mass spectra. Furthermore, abnormal product ions containing a pyrogallol (P) group ($[P–H]^+$/$[P–3H]^-$) with loss of two hydrogen atoms compared with the normal product ions ($[P+H]^+$/$[P–H]^-$) were also found in the mass spectra. These abnormal product ions, generated from the suspected oxidation product of baicalin, were produced during the collision-induced dissociation (CID) process.

Specifically, with positive ESI-MS, baicalin yielded a normal protonated precursor ion *m/z* 447 ($[M+H]^+$). At the same time, an abnormal precursor ion *m/z* 445 ($[M–H]^+$) was unambiguously observed (Fig. 1), which probably arose through the loss of two hydrogen atoms compared with the normal precursor ion. This speculation was based on the deduced chemical formula ($C_{21}H_{17}O_{11}$) from the observed accurate mass (*m/z* 445.0741). Therefore, the calculated chemical formula of the compound corresponding to the ion *m/z* 445 was $C_{21}H_{16}O_{11}$, which presumably was an oxidized product of baicalin with an *ortho*-diquinone group in its structure as illustrated in Fig. 1 (baicalin oxide). In addition, at the retention time of baicalin, a product ion at *m/z* 271.0560 was found (Fig. 1), corresponding to the precursor ion of baicalein based on the calculated chemical formula ($C_{15}H_{11}O_5$) from the detected accurate mass. This ion was generated by baicalin during the CID process by losing a glucuronide (G) group ($[M–G+H]^+$). Adjacent to

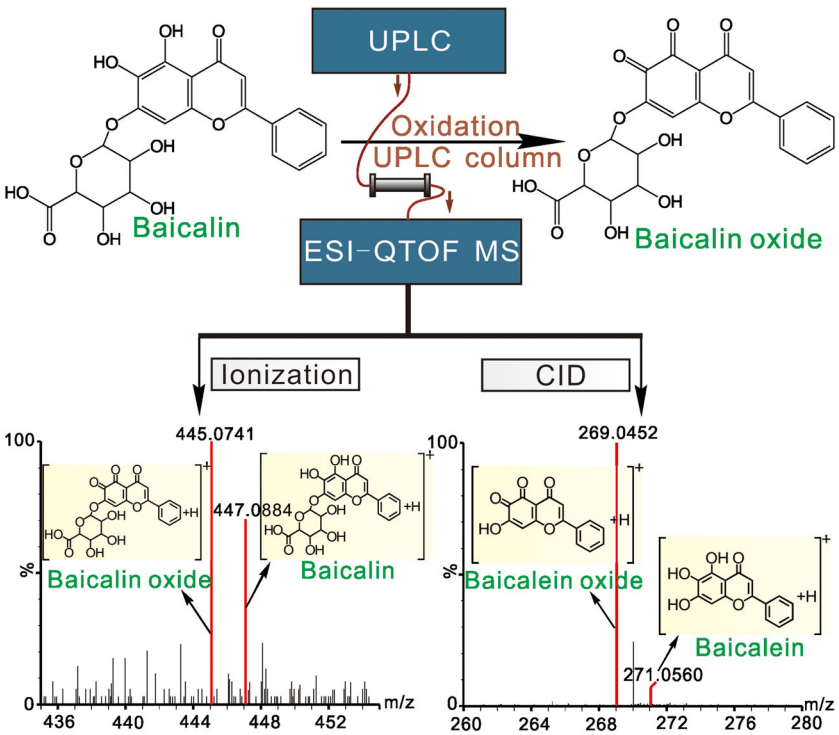
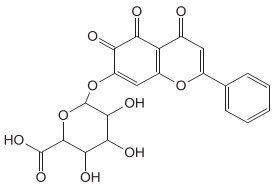
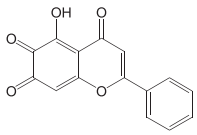
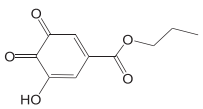
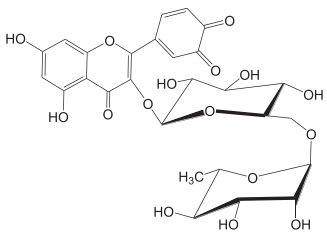
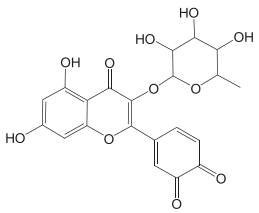



**Figure 1.** Schematic diagram of probable oxidation reaction process on a UPLC column and detection with high-resolution mass spectrometry. Baicalin could generate the precursor ion of baicalein via a collision-induced dissociation (CID) process.



**Table 1.** Proposed chemical formulas, structures and theoretical exact molecular weights of the on-column oxidized products of the investigated model compounds

| Name | Molecular formula | Exact molecular weight (u) | Chemical structure |
|---|---|---|---|
| Baicalin oxide | $C_{21}H_{16}O_{11}$ | 444.0693 |  |
| Baicalein oxide | $C_{15}H_8O_5$ | 268.0372 |  |
| PG oxide | $C_{10}H_{10}O_5$ | 210.0529 |  |
| Rutin oxide | $C_{27}H_{28}O_{16}$ | 608.1377 |  |
| QR oxide | $C_{21}H_{18}O_{11}$ | 446.0849 |  |

PG: Propyl gallate; QR: quercetin-3-rhamnoside.

this ion, another product ion at *m/z* 269.0452 was also present in the mass spectrum. This product ion (*m/z* 269.0452) was inferred to be the precursor ion of baicalein with loss of two hydrogen atoms ($[M–G–H]^+$) according to the calculated chemical formula ($C_{15}H_9O_5$). We deduced that this product ion (*m/z* 269.0452) would correspond to an oxidation product of baicalein with an *ortho*-diquinone group (Fig. 1), which was generated during the CID process from the precursor ion of a possible oxidation product of baicalin (*m/z* 445.0741) by loss of a G group. It is suggested that oxidation reactions of polyphenolic compounds such as baicalin could take place on a UPLC/ESI-MS system based on the observation of the abnormal ions *m/z* 445 ($[M–H]^+$) and *m/z* 269 ($M–G–H]^+$), corresponding to possible oxidized products of baicalin and baicalein, respectively.

Abnormal precursor ions and product ions were observed for other model compounds including baicalein, PG, rutin and QR, indicating that oxidation reactions of these polyhenolic compounds could also happen in a UPLC/ESI-MS system. The theoretical exact masses, detected accurate masses, mass accuracy (ppm), and calculated chemical formulas of the precursor ions of the model

compounds and their possible oxidation derivatives are documented in Supplementary Table S5 (see Supporting Information).

### Ascertaining the oxidation reaction on the UPLC/ESI-MS system

In order to ascertain the oxidation reactions occurring on the UPLC/ESI-MS system, post-column infusion of reducing agent was further performed. The operating details are illustrated in Fig. 2. The tested reducing agents included $(NH_4)_2S$, VC, hydroquinone, catechol, PG, dopamine, *ortho*-phenylenediamine, phloroglucinol, formaldehyde, and resorcinol. Upon post-column infusion of $(NH_4)_2S$ (1.7 mM) at 50 μL/min, the ion *m/z* 445 almost disappeared in the mass spectrum, whereas *m/z* 447, the precursor ion of baicalin, was still present with even enhanced signal intensity (Fig. 2). In addition, the ratio of the signal intensity of *m/z* 269 (possible oxidized derivative of baicalein) to that of *m/z* 271 (baicalein) was significantly decreased (Fig. 2). Similar results were obtained with other reducing agents such as VC and PG. These results confirmed that an oxidization reaction really occurred on the UPLC/ESI-MS system.

In addition, $(NH_4)_2S$ was found to be the most suitable reducing agent due to its significant reducing efficiency and negligible ion suppression for ESI-MS operated in both positive and negative modes. Moreover, $(NH_4)_2S$ was found to be capable of enhancing the signal intensity of the analytes in positive ESI-MS mode. Therefore, $(NH_4)_2S$ was finally chosen as the reducing agent to perform further post-column infusion.

### Confirmation of the oxidation reaction on the UPLC column

It is well known that an ESI source can be considered as an electrolytic cell where redox reactions may occur via electrolysis.[7,8] Previous studies suggested that electrochemical oxidation reactions occurred only in a positive ESI source and electrochemical reduction reactions took place only in a negative ESI source.[7,11,12] In this study, however, oxidization reactions were observed not only with positive ESI-MS, but also with negative ESI-MS without significant difference in the extent of reactions (Fig. 1 and Supplementary Fig. S2, see Supporting Information). Therefore, post-column infusion of a baicalin solution was conducted in order to investigate whether the oxidation happened in the ESI source or on the UPLC columns (Fig. 3).

Baicalin solution (20 ng/mL in water) was infused at a flow rate of 10 μL/min. In the mass spectrum of *m/z* 435–455 at the retention time range of baicalin (0.8–0.9 min), both the precursor ions of baicalin (*m/z* 447) and baicalin oxide (*m/z* 445) were present. The signal intensity of the ion at *m/z* 445 was even stronger than that of the ion at *m/z* 447 (Fig. 3). However, in the retention time ranges other than that of baicalin (such as 0.1–0.2 min), only the precursor ion of baicalin (*m/z* 447) was found, whereas the precursor ion of its oxide (*m/z* 445) was undetectable. Post-column infused baicalin solution was directly transferred to the ESI source without passing through the UPLC column. This result demonstrated that baicalin was not oxidized during the ESI process. When a blank solution (MeOH/$H_2O$, 1:1, v/v) was injected onto the UPLC/MS system accompanied by post-column infusion of baicalin solution, in the mass spectra of *m/z* 435–455 and *m/z* 268.5–273

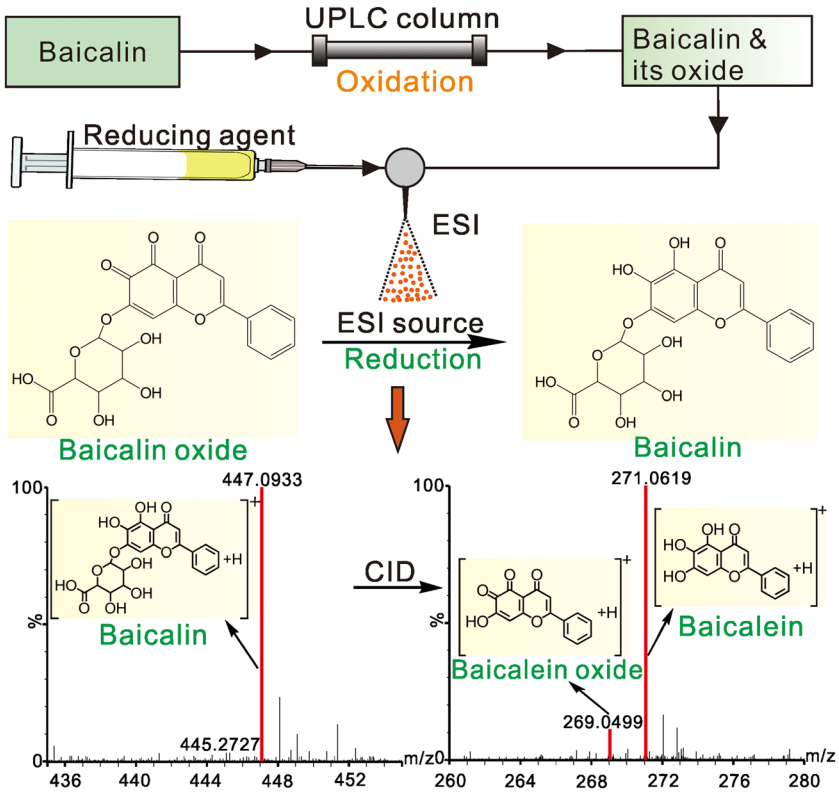



**Figure 2.** Oxidation reaction on UPLC column and reduction reaction process in ESI source with the post-column infusion of reducing agent solutions. The mainly used reducing agent was $(NH_4)_2S$.



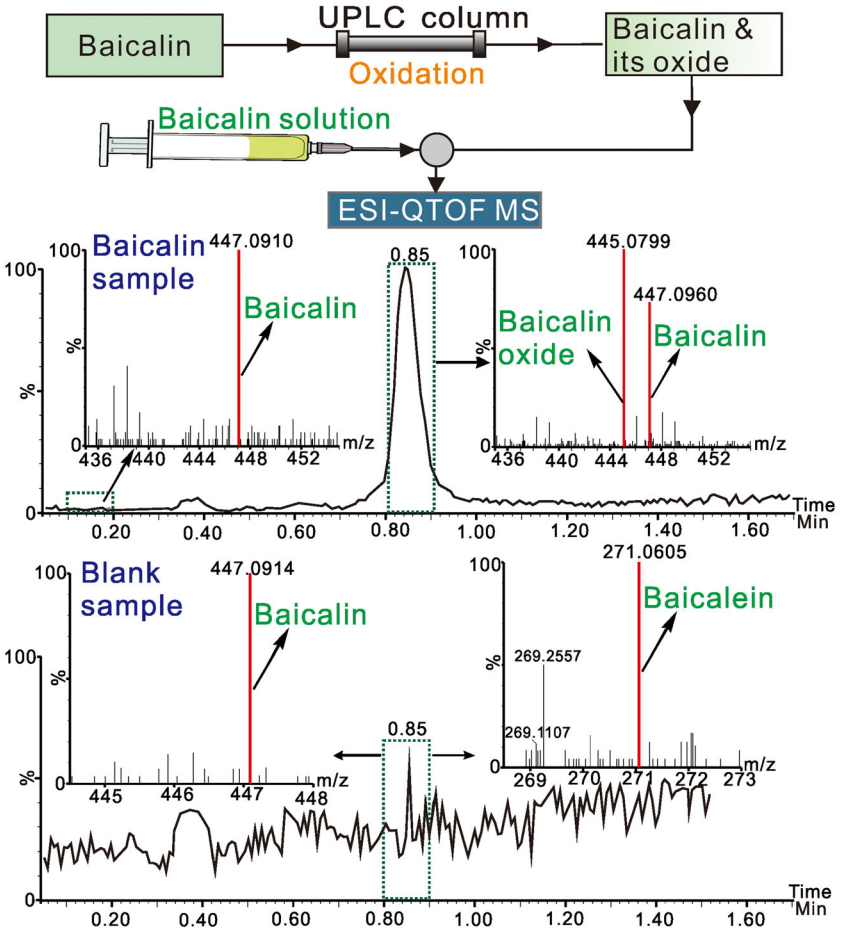



**Figure 3.** Confirmation for on-column oxidation reaction with post-column infusion of baicalin solution. The chromatographic gradient elution program I was used (Supplementary Table S2, see Supporting Information).

in the retention time range of baicalin (0.8–0.9 min), only the precursor ions of baicalin (*m/z* 447) and baicalein (*m/z* 271) were observed, whereas the precursor ions of baicalin oxide (*m/z* 445) and baicalein oxide (*m/z* 269) were not detected. This result elucidated that chemical impurities which were presumptively capable of oxidizing baicalin and baicalein in the ESI source were ineffective on the oxidation reaction.

The above-mentioned results and discussion confirmed that the oxidation reactions observed in this study occurred on the UPLC columns rather than in the ESI source.

### Exact site of the on-column oxidation reaction

The majority of the research concerning degradation of compounds on HPLC and UPLC columns has focused on degradation of macromolecules such as proteins, DNA, and polymers.[30–32] Only two studies have, so far, reported on-column (HPLC) degradation of small molecules.[28,29] Wang *et al.*[28] considered that the on-column dimerization of anilines was a surface reaction caused by oxidation agents that were generated during complexation between unknown elements on the stationary phase surface of the X-Bridge LC columns and ammonia in the high-pH mobile phase. Myers *et al.*[29] suggested that the on-column nitrosation of amines occurred at the stainless steel frit of the HPLC column and ablated metals of the frit. Degradation products observed in these two studies were chromatographically separable and thus could be discriminated using HPLC ultraviolet–visible (UV) detection. In our study, however, the oxidation reaction could hardly be observed on common HPLC columns due to the low extent of the reactions. As shown in Supplementary Fig. S3 (see Supporting Information), only an inconspicuous oxidation reaction was observed on two types of HPLC columns [Ultimate® AQ-C18 column (4.6 × 150 mm, 3 µm; Welch Materials, Shanghai, China) and XBridge™ C8 column (4.6 × 250 mm, 5 µm; Waters, Milford, MA, USA)]. In contrast, an evident oxidation reaction was observed on the 1.8-µm UPLC columns in this study. In addition, the oxidized products could not be chromatographically separated from their parent compounds with similar peak shapes, particularly for baicalin and baicalin oxide (Fig. 4). If the oxidization reaction occurred on the inlet frit, the oxidation products would be chromatographically separable from their parent compounds with independent peaks, as observed by Wang *et al.*[28] and Myers *et al.*[29] If the reaction took place on the stationary phase surface of the UPLC columns, the oxidation process could have occurred continually during the retention time ranges of the model compounds, giving rise to distorted and broadened chromatographic peaks for the oxidized products. Therefore, the oxidation reactions did not occur at either the inlet frits or on the stationary phase surface of the UPLC columns. Thus, the outlet frits of the UPLC columns

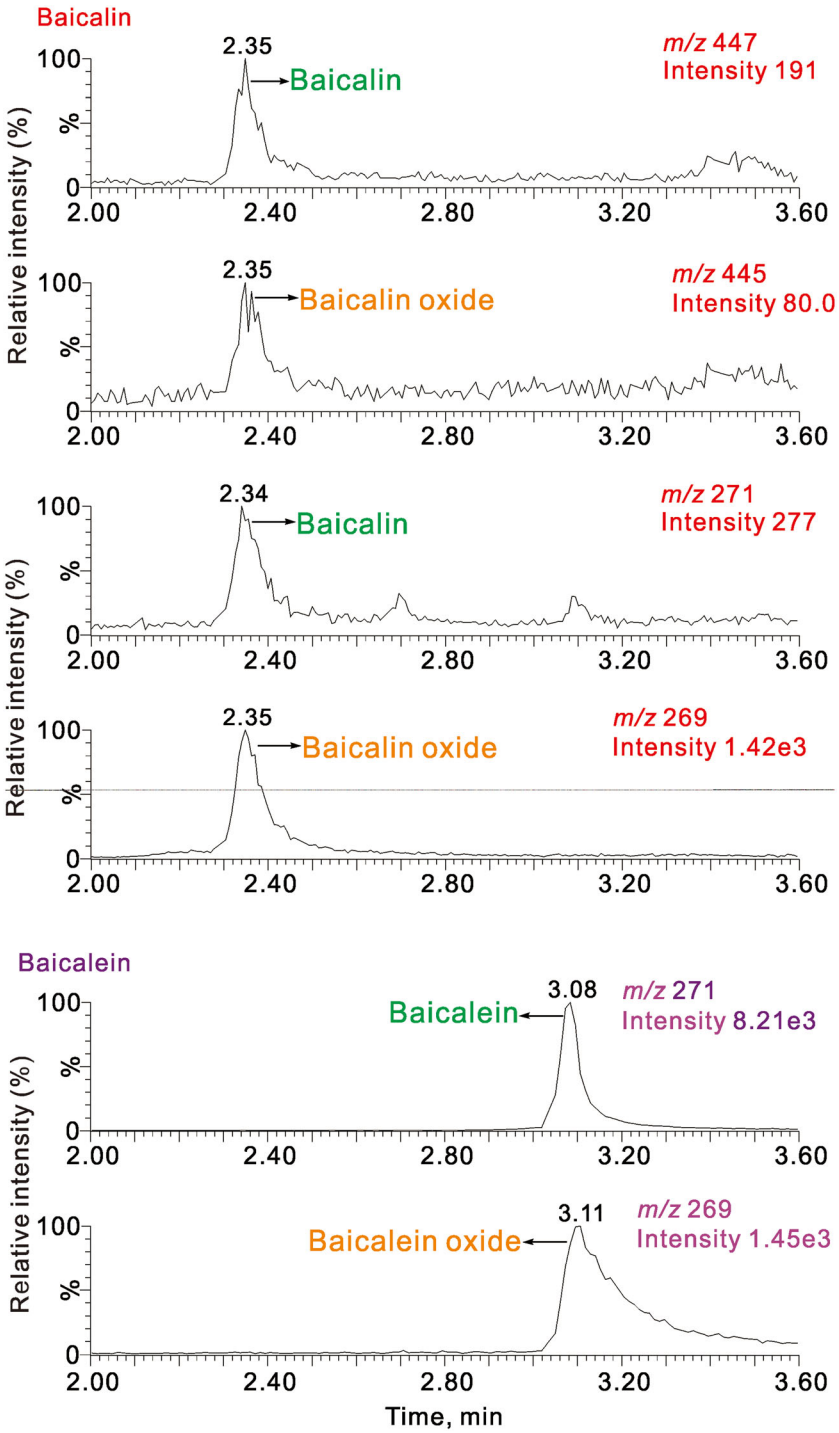



**Figure 4.** Representative chromatograms of baicalin, baicalein and their oxidized products caused by on-column oxidation reaction. The chromatographic gradient elution program II was used (Supplementary Table S3, see Supporting Information).

should be the most reasonable site for the oxidation reactions to take place according to the identical or similar retention times and peak shapes of the oxidized products to their parent compounds. The chromatograms of baicalin, baicalein and their oxidized products are provided in Fig. 4.

To further confirm this speculation, a UPLC+UPLC experiment was conducted. As shown in Fig. 5, the retention times of baicalin, baicalin oxide, baicalein, and baicalein oxide were 2.87, 2.50, 7.21 and 4.33 min, respectively, indicating these compounds were chromatographically separable on UPLC columns, particularly for baicalein and baicalein oxide. In addition, at the retention times of baicalin and baicalein (2.87 and 7.21 min), the corresponding oxidation products (baicalin oxide and baicalein oxide) were also observed, which means that the oxidation products could be chromatographically separated from their respective parent compounds. These results indicated that the outlet frits were the exact sites where the oxidation reaction took place. In



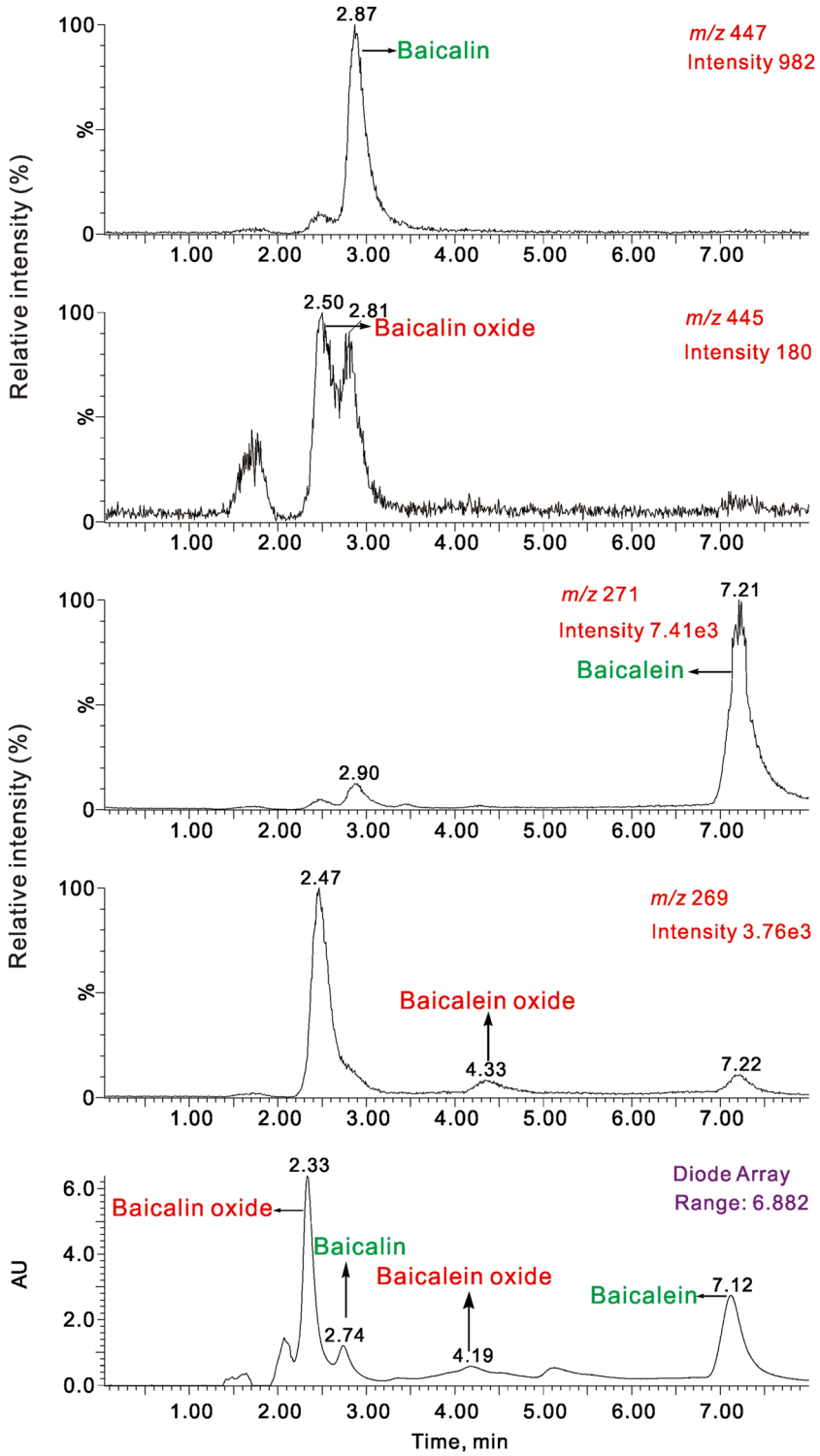



**Figure 5.** Chromatograms of baicalin, baicalein and their on-column oxidized products on two coupled UPLC columns (UPLC+UPLC). The first UPLC column was Waters BEH C18 column (2.1×50 mm, 1.7 μm) and the second was Waters HSS T3 column (2.1×100 mm, 1.8 μm). Isocratic elution program with ACN/$H_2O$ (4:6, *v/v*) with the flow rate of 300 μL/min was used.

addition, four peaks at 2.33, 2.74, 4.19, and 7.12 min were observed in the chromatogram with UV diode-array detection, corresponding to baicalin oxide, baicalin, baicalein oxide and baicalein, respectively (Fig. 5). The extracted UV-spectra at these retention times showed evidently different characteristic absorption patterns at 2.74 and 7.12 min from those at 2.33 and 4.19 min, respectively (Supplementary Fig. S4, see Supporting Information). In addition, the maximum absorption wavelength at 4.19 min was shorter (hypsochromic effect) than that at 7.12 min (Supplementary Fig. S4, see

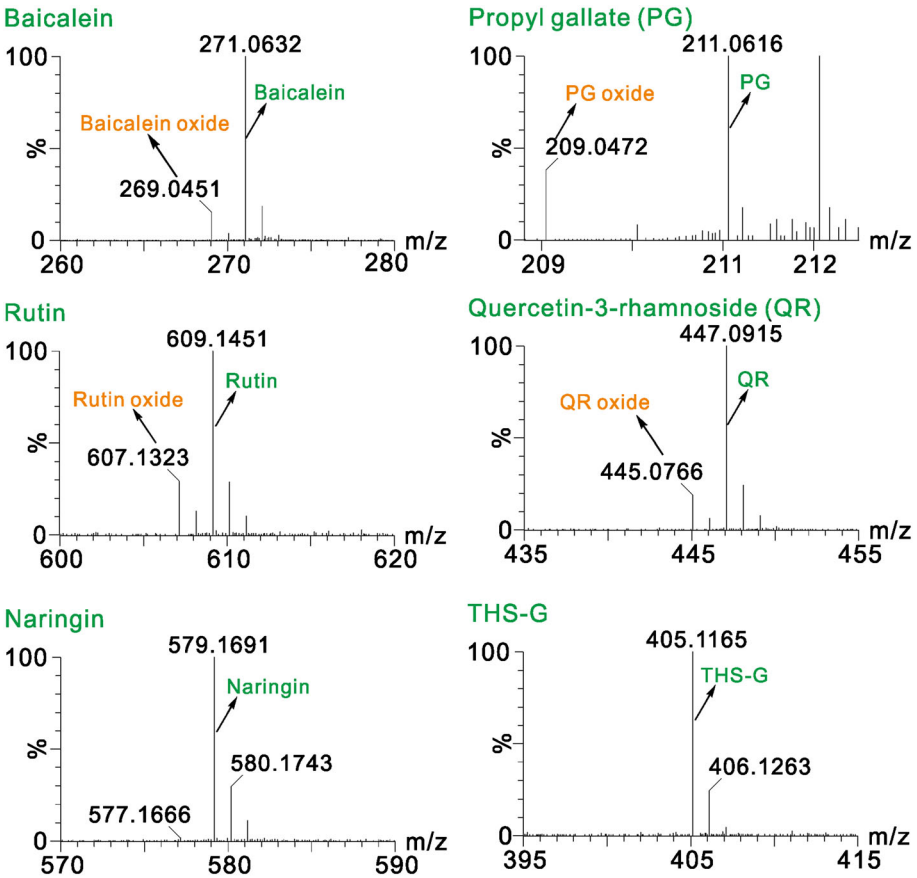



**Figure 6.** High resolution mass spectra of the investigated model compounds (excepted baicalin) and their oxidized products caused by on-column oxidation reaction. Baicalein was detected with positive ESI source, and others were detected with negative ESI source.

Supporting Information). These phenomena also supported the conclusion that the oxidation reactions occurred at the outlet frits of the UPLC columns.

Frit-removed experiments were further carried out to confirm the exact site of the oxidations. First, the inlet frit was removed from the UPLC column that was used for separation. As illustrated in Supplementary Fig. S5 (see Supporting Information), baicalin oxide presented relatively high signal intensity, indicating a high extent of oxidation reaction. This result demonstrated the on-column oxidation reaction could happen without the inlet frit. In the following step, both the inlet and outlet frits were removed from the UPLC column. The result showed that the signal intensity of baicalin oxide decreased dramatically, indicating significantly reduced extent of the oxidation reaction (Supplementary Fig. S5, see Supporting Information). This result revealed that the on-column oxidation reaction could not happen without the outlet frit.

### Tentative mechanistic considerations

Shear force and extensional force have been considered as the main causes for scission of polymer chains and biomacromolecules on chromatographic columns.[24] According to the shear rate calculation,[24,25,33] the shear rate generated in the UPLC columns and that on the frits (0.2 μm) were estimated to be $7 \times 10^4\ S^{-1}$ and $2 \times 10^5\ S^{-1}$, respectively, in this study, which were high enough to cause scission between C–C bonds of the polymers and other covalent bonds of macromolecules.[24,25] Therefore, the high shear rates at the outlet frits of the UPLC columns were postulated to facilitate the cleavage of the H–O bonds on the phenol ring, and thus generated quinones and $H_2O$ with the presence of oxidation agents such as $O_2$ and OH• in the mobile phases. All reactions might be catalyzed by the active metals of the frits. In addition, surface effect might also play a role in the oxidation process.

An experiment using a serial of elution flow rates, i.e., 50, 200, 300, 400, and 600 μL/min, was conducted to validate the above hypotheses. As shown in Supplementary Fig. S6 (see Supporting Information), the on-column oxidation reaction did not change significantly with the variation in the elution flow rates. On the contrary, the oxidation reaction was relatively stronger at the flow rate of 50 μL/min. This observation does not agree with the "shear degradation" theory by which shear rate is positively proportional to flow rate.[24,34,35] Therefore, "shear degradation" is not a plausible explanation for the on-column oxidation reaction observed in this work. In addition, according to the information provided by the manufacturer of the tested UPLC columns, the pore sizes and materials of the inlet and outlet frits are identical, which means that the shear forces generated on both the inlet frits and the outlet frits should be equivalent. According to the "shear degradation" assumption,[24] the on-column oxidation reactions should have been observed



on both the inlet and the outlet frits. Therefore, "shear degradation" was not the probable mechanism of the on-column oxidation observed in this study.

A surprising phenomenon was observed when a UPLC column was used in reversed flow direction for separation. With this condition, the on-column oxidation reaction completely disappeared (Supplementary Fig. S7, see Supporting Information). Unfortunately, no convincible interpretation can be made based on available data and knowledge.

To sum up, the oxidation reactions occurring on UPLC columns could be attributable to metal catalysis, surface effect and other unknown factors. Further efforts are needed to explore the mechanisms of the on-column oxidation reactions.

### On-column oxidation for other compounds

On-column oxidation reactions were also observed for baicalein, PG, rutin, and QR (Fig. 6). The proposed chemical structures, formulas, and calculated molecular weights of the oxidized products are provided in Table 1. For naringin and THS-G, however, an oxidation reaction was not found on the tested UPLC columns. These two compounds, possessing either a *para*-hydroxybenzene group or a resorcinol group in their structures, may have relatively lower reducing property compared with the other polyphenolic compounds.

### Finding the most suitable reducing agent

In this study, $(NH_4)_2S$ was found to be the most effective and suitable reducing agent in eliminating unnecessary oxidation reaction on UPLC columns (Supplementary Figs. S8–S11, see Supporting Information). Thus $(NH_4)_2S$ was proposed to be a useful agent for UPLC/MS analysis to counteract oxidation reactions and ensure analyzing accuracy. Other reducing agents including hydroquinone, dopamine, catechol, *ortho*-phenylenediamine, PG, and routinely used VC could also convert on-column oxidized products into their parent forms in the ESI source. However, their reducing efficiencies were remarkably lower than that of $(NH_4)_2S$. In addition, these reducing agents could suppress the ionization efficiency of the target analytes to some extent in either positive or negative mode in the ESI source. Moreover, they are in possession of relatively higher molecular weights and more difficult to be decomposed in comparison with $(NH_4)_2S$, which could lead to contamination in the ESI source.

### Applications

Natural product chemistry has become a hot spot in research areas of chemistry, plant science, and pharmaceutical sciences.[36–39] Among numerous natural products, polyphenolic substances and flavonoids have attracted particular attention due to their benefits to human health.[40–42] Identification and quantification of natural products are of priority for further studies. The oxidation reaction of polyphenolic compounds possibly occurring on UPLC columns may cause biased results. Therefore, precautions should be taken to eliminate possible on-column oxidation reactions for qualitative and quantitative analysis. Post-column infusion of reducing agents can transform the oxidized products back to their parent compounds. Direct addition of reducing reagent into mobile phases could help to suppress on-column oxidation reactions.

## CONCLUSIONS

Oxidation reactions of some polyphenolic compounds occurring on UPLC columns were observed and confirmed through UPLC/HRMS detection and post-column infusion. Oxidation was confirmed to occur at the outlet frits of the UPLC columns. The on-column oxidation can be compensated by post-column infusion of reducing reagent into the ESI source. This work will be an enlightenment for identification and quantification of natural products as polyphenolic compounds and quinonoids using UPLC/MS.

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## REFERENCES

[1] R. Plumb, J. Castro-Perez, J. Granger, I. Beattie, K. Joncour, A. Wright. Ultra-performance liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 2331.
[2] N. L. Kuehnbaum, P. Britz-McKibbin. New advances in separation science for metabolomics: resolving chemical diversity in a post-genomic era. *Chem. Rev.* **2013**, *113*, 2437.
[3] C. M. Tang, C. X. Tang, W. Zhan, H. Zheng, Z. F. Wang, X. Z. Peng. Strategies for ascertaining the interference of phase II metabolites co-eluting with parent compounds using LC-MS/MS. *J. Sep. Sci.* **2013**, *36*, 2584.
[4] I. L. Kanev, A. Y. Mikheev, Y. M. Shlyapnikov, E. A. Shlyapnikova, T. Y. Morozova, V. N. Morozov. Are reactive oxygen species generated in electrospray at low currents? *Anal. Chem.* **2014**, *86*, 1511.
[5] S. P. Pasilis, V. Kertesz, G. J. Van Berkel. Unexpected analyte oxidation during desorption electrospray ionization-mass spectrometry. *Anal. Chem.* **2008**, *80*, 1208.
[6] B. L. Boys, M. C. Kuprowski, J. J. Noe, L. Konermann. Protein oxidative modifications during electrospray ionization: solution phase electrochemistry or corona discharge-induced radical attack? *Anal. Chem.* **2009**, *81*, 4027.
[7] S. Plattner, R. Erb, J. P. Chervet, H. Oberacher. Ascorbic acid for homogenous redox buffering in electrospray ionization-mass spectrometry. *Anal. Bioanal. Chem.* **2012**, *404*, 1571.
[8] E. Peintler-Krivan, G. J. Van Berkel, V. Kertesz. Minimizing analyte electrolysis in electrospray ionization mass spectrometry using a redox buffer coated emitter electrode. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1327.

  

[9] E. Peintler-Krivan, G. J. Van Berkel, V. Kertesz. Poly-(3,4-ethylenedioxypyrrole)-modified emitter electrode for substitution of homogeneous redox buffer agent hydroquinone in electrospray ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 3368.
[10] R. D. Espy, M. Wleklinski, X. Yan, R. G. Cooks. Beyond the flask: Reactions on the fly in ambient mass spectrometry. *TrAC, Trends Anal. Chem.* **2014**, *57*, 135.
[11] D. W. Looi, I. Iftikhar, A. Brajter-Toth. Electrochemical attributes of electrochemistry in tandem with electrospray mass spectrometry. *Electroanalysis* **2014**, *26*, 319.
[12] S. Plattner, R. Erb, J. P. Chervet, H. Oberacher. Studying the reducing potencies of antioxidants with the electrochemistry inherently present in electrospray ionization-mass spectrometry. *Anal. Bioanal. Chem.* **2014**, *406*, 213.
[13] H. Oberacher, F. Pitterl, R. Erb, S. Plattner. Mass spectrometric methods for monitoring redox processes in electrochemical cells. *Mass Spectrom. Rev.* **2015**, *34*, 64.
[14] F. Qin, Y. Y. Zhao, Y. Zhao, J. M. Boyd, W. Zhou, X. F. Li. A toxic disinfection by-product, 2,6-dichloro-1,4-benzoquinone, identified in drinking water. *Angew. Chem. Int. Ed.* **2010**, *49*, 790.
[15] Y. Zhao, F. Qin, J. M. Boyd, J. Anichina, X. F. Li. Characterization and determination of chloro- and bromobenzoquinones as new chlorination disinfection byproducts in drinking water. *Anal. Chem.* **2010**, *82*, 4599.
[16] W. Wang, Y. Qian, J. Li, B. Moe, R. Huang, H. Zhang, E. H. Steve, X. F. Li. Analytical and toxicity characterization of halo-hydroxyl-benzoquinones as stable haloquinone disinfection byproducts in treated water. *Anal. Chem.* **2014**, *86*, 4982.
[17] S. L. Isenberg, A. K. Brewer, G. L. Côté, A. M. Striegel. Hydrodynamic versus size exclusion chromatography characterization of alternan and comparison to off-line MALS. *Biomacromolecules* **2010**, *11*, 2505.
[18] A. M. Striegel, S. L. Isenberg, G. L. Côté. An SEC/MALS study of alternan degradation during size-exclusion chromatographic analysis. *Anal. Bioanal. Chem.* **2009**, *394*, 1887.
[19] J. Bao, S. M. Krylova, L. T. Cherney, J. Y. LeBlanc, P. Pribil, P. E. Johnson, D. J. Wilson, S. N. Krylov. Kinetic size-exclusion chromatography with mass spectrometry detection: An approach for solution-based label-free kinetic analysis of protein-small molecule interactions. *Anal. Chem.* **2014**, *86*, 10016.
[20] A. M. Striegel. Observations regarding on-column, flow-induced degradation during SEC analysis. *J. Liq. Chromatogr. Rel. Technol.* **2008**, *31*, 3105.
[21] R. A. Cave, S. A. Seabrook, M. J. Gidley, R. G. Gilbert. Characterization of starch by size exclusion chromatography: The limitations imposed by shear scission. *Biomacromolecules* **2009**, *10*, 2245.
[22] A. K. Brewer, A. M. Striegel. Characterizing string-of-pearls colloidal silica by multidetector hydrodynamic chromatography and comparison to multidetector size-exclusion chromatography, off-line multiangle static light scattering, and transmission electron microscopy. *Anal. Chem.* **2011**, *83*, 3068.
[23] H. G. Barth, F. J. Carlin. A review of polymer shear degradation in size-exclusion chromatography. *J. Liq. Chromatogr.* **1984**, *7*, 1717.
[24] E. Uliyanchenko, S. Van der Wal, P. J. Schoenmakers. Deformation and degradation of polymers in ultra-high-pressure liquid chromatography. *J. Chromatogr. A* **2011**, *1218*, 6930.
[25] A. M. Striegel. Do column frits contribute to the on-column, flow-induced degradation of macromolecules? *J. Chromatogr. A* **2014**, *1359*, 147.
[26] N. Häring, K. Ballschmiter. Chromatographie von metallchelaten-IX adsorptive voranreicherung für bestimmungen von kobalt, kupfer und nickel im mikrogramm/liter-bereich nach umkehrphasen-chromatographie der diäthyldithiocarbamate. *Talanta* **1980**, *27*, 873.
[27] P. Heizmann, K. Ballschmiter. Chromatography of metal chelates: VII. High-performance liquid chromatography of metal 1,2-diketobisthiobenzhydrazones, metal dialkyldithiocarbamates and metal 1,2-diketobisthiosemicarbazones. *J. Chromatogr.* **1977**, *137*, 153.
[28] F. Wang, X. K. Liu, S. Lai, J. Fang, D. Semin. Chromatographic studies of unusual on-column degradations of aniline compounds on XBridge Shield RP18 column in high pH aqueous mobile phase. *J. Chromatogr. A* **2011**, *1218*, 3502.
[29] D. P. Myers, E. M. Hetrick, Z. Liang, C. E. Hadden, S. Bandy, C. A. Kemp, T. M. Harris, S. W. Baertschi. On-column nitrosation of amines observed in liquid chromatography impurity separations employing ammonium hydroxide and acetonitrile as mobile phase. *J. Chromatogr. A* **2013**, *1319*, 57.
[30] J. Hirabayashi, K. Kasai. Slalom chromatography: A new size-dependent separation method for DNA. *Nucleic Acids Symp. Ser.* **1988**, *20*, 67.
[31] B. E. Boyes, D. G. Walker, P. L. McGeer. Separation of large DNA restriction fragments on a size-exclusion column by a nonideal mechanism. *Anal. Biochem.* **1988**, *170*, 127.
[32] Y. G. Liu, W. Radke, H. Pasch. Coil-stretch transition of high molar mass polymers in packed-column hydrodynamic chromatography. *Macromolecules* **2005**, *38*, 7476.
[33] R. B. Bird, W. E. Stewart, E. N. Lightfoot. *Transport Phenomena*. John Wiley, New York, **1960**.
[34] P. J. Schoenmakers, P. J. Aarnoutse. Multi-dimensional separations of polymers. *Anal. Chem.* **2014**, *86*, 6172.
[35] E. Uliyanchenko, P. J. Cools, S. van der Wal, P. J. Schoenmakers. Comprehensive two-dimensional ultrahigh-pressure liquid chromatography for separations of polymers. *Anal. Chem.* **2012**, *84*, 7802.
[36] G. M. Cragg, P. G. Grothaus, D. J. Newman. Impact of natural products on developing new anti-cancer agents. *Chem. Rev.* **2009**, *109*, 3012.
[37] V. De Luca, V. Salim, S. M. Atsumi, F. Yu. Mining the biodiversity of plants: A revolution in the making. *Science* **2012**, *336*, 1658.
[38] D. J. Newman, G. M. Cragg. Natural products as sources of new drugs over the last 25 years. *J. Nat. Prod.* **2007**, *70*, 461.
[39] F. E. Koehn, G. T. Carter. The evolving role of natural products in drug discovery. *Nat. Rev. Drug Discov.* **2005**, *4*, 206.
[40] K. C. Nicolaou, Q. Kang, T. R. Wu, C. S. Lim, D. Y. K. Chen. Total synthesis and biological evaluation of the resveratrol-derived polyphenol natural products hopeanol and hopeahainol A. *J. Am. Chem. Soc.* **2010**, *132*, 7540.
[41] I. Tejero, N. González-García, À. González-Lafont, J. M. Lluch. Tunneling in green tea: understanding the antioxidant activity of catechol-containing compounds. A variational transition-state theory study. *J. Am. Chem. Soc.* **2007**, *129*, 5846.
[42] D. Y. Xie, S. B. Sharma, N. L. Paiva, D. Ferreira, R. A. Dixon. Role of anthocyanidin reductase, encoded by BANYULS in plant flavonoid biosynthesis. *Science* **2003**, *299*, 396.

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