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Identification and Characterization of Polyphenolic Antioxidants Using On-Line Liquid Chromatography, Electrochemistry, and Electrospray Ionization Tandem Mass Spectrometry

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It is demonstrated that electrochemistry (EC) coupled to liquid chromatography (LC) and electrospray ionization tandem mass spectrometry (LC/EC/ESI-MS/MS) can be used to rapidly obtain information about the antioxidant activity (i.e., oxidation potential) and capacity (i.e., amount) of polyphenolic compounds, including catechin, kaempferol, resveratrol, quercetin, and quercetin glucosides. The described on-line LC/EC/ESI-MS/MS method facilitates the detection and characterization of individual antioxidants based on a combination of the obtained *m/z* values for the antioxidants and their oxidation products, the potential dependences for the ion intensities, and correlations between the retention times in the LC, EC, and MS chromatograms. As these results provide patterns that can be used in rapid screening for antioxidants in complex samples, the method should be a valuable complement to chemical assays commonly used to determine the total antioxidant capacity of samples. It is shown that the antioxidant capacity for a mixture of polyphenolic compounds depends on the redox potential employed in the evaluation, and this should consequently be taken into account when comparing results from different total antioxidant capacity assays. It is also demonstrated that the inherent antioxidant capacities of phenolic compounds increase with an increasing number of hydroxyl groups and that the potential needed to oxidize the remaining hydroxyl groups increases successively upon oxidation of the compound. Unlike chemical assays, which generally do not provide any information about the identities of the compounds on the molecular level, the present screening method can be used to identify individual antioxidants, rank compounds with respect to their ease of oxidation, and to study the antioxidant capacity at any redox potential of interest.

Within the rapidly expanding field of antioxidant research, there is currently a need for novel tools that facilitate fast and simple screening of compounds with antioxidant properties. There is likewise a demand for methods that can be used to rapidly assess the antioxidant activity (ease of oxidation) and capacity (amount) on the molecular level for individual compounds present in complex samples as a complement to currently employed chemical assays in which the total capacity of a sample containing many antioxidants is evaluated.

Antioxidants, also called free-radical scavengers, which are reactive as hydrogen- or electron-donating agents[1] can be studied by chemical and/or electrochemical methods. There are many different chemical assays[2−9] that can be utilized to determine the total antioxidant capacity (TAC) of a sample. These assays can be classified into two main categories, the hydrogen atom transfer and the single electron transfer reaction based assays.[3] The chemical assays, however, have some limitations, since the total capacity, and not individual antioxidant capacity of the specific compounds, is determined. The latter makes identifications and extractions of interesting antioxidants from samples difficult. Furthermore, the chemicals used in the assays are generally expensive and the assays are often both tedious and time-consuming to perform.

To address some of the limitations with conventional chemical assays, a number of high -resolution antioxidant screening methods (HRS), recently reviewed by Niederländer et al.,[10] have been evaluated. In HRS, chromatographic separation is combined mainly with a chemical assay detection step, to facilitate screening

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of individual antioxidants in complex samples. The applicability of this approach is, however, limited by the fact that it is challenging to preserve the chromatographic resolution on the time-scale of the chemical assays. With the commonly employed free radical discoloration assay based on the stable free radical 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•),[8,10] an on-line approach is also complicated[11,12] by the fact that this assay is sensitive to variations in the composition and pH of the mobile phase.

As has been previously demonstrated, antioxidants can also be studied using electrochemical (EC) methods[10,13−15] with which the above-mentioned limitations are less of a problem. As antioxidants are compounds which readily undergo oxidation, determinations of the oxidation potentials can be employed to investigate the antioxidant activity while the antioxidant capacity can be obtained from the oxidation charges. A low oxidation potential indicates that the compound is easily oxidized and thereby may act as a good antioxidant.[1,16,17] The antioxidant capacity is determined by the amount of the antioxidant in the sample and the number of oxidizable groups present on the antioxidant. Results obtained in EC measurements have been shown to have some correlation with results obtained with chemical assays[10,14,18,19] if an appropriate oxidation potential is selected. However, the correlation between EC results and those obtained from other assays is still not clear. Although electrochemical measurements are rapid and straightforward, these techniques are, however, less applicable when complex samples containing many electroactive compounds are to be analyzed. There is thus a need for new methods, involving combinations of electrochemistry and other techniques that can deal with the latter problem.

Upon analysis of complex samples, an identification of the individual compounds generally requires an approach involving chromatography and mass spectrometric detection. An electrochemical cell can also be included in the system to enable the detection of electrochemically active species. Dewald et al.[20] were the first to use LC/EC/TSP-MS for separation and identification of isoflavones. In 2001, Diehl and co-workers[21] described the first on-line combination of LC/EC/MS using either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) interfaces for the study of ferrocenecarboxylic acid esters of various alcohols and phenols. In 2002, Jurva et al.[22] introduced an EC flow-through system that allowed the generation of hydroxyl radicals for reaction with xenobiotics and subsequent detection of the oxidation products on-line with LC/MS/MS. It was found that the system was useful for investigations of new radical scavengers and antioxidants. The use of LC on-line with a chemical assay and MS has recently been reported by several research groups.[23−26]

Polyphenols or phenolic compounds are well-known antioxidants, and the presence of polyphenols in food is becoming increasingly recognized as important to long-term health and the reduction of the risk of chronic diseases.[27] Phenolic compounds, for example, flavonoids, which can be found in beverages, vegetables, and fruit, thus contribute to the antioxidant capacity of the food.[1] The flavonoids constitute a large class of compounds, which can be subdivided into several families; flavonols (e.g., quercetin and kaempferol), flavanols (e.g., catechin), flavones, and isoflavones.[1] Beverages such as green and black teas and red wine contain catechin, quercetin, and kaempferol.[2] In wine another antioxidant, resveratrol, can also be found,[28−31] whereas quercetin and kaempferol can be found in onions.[32−34]

In the present study, the use of an on-line LC/EC/ESI-MS/MS method for rapid screening of antioxidants is described. By separation of the sample components in a mixture using LC prior to their oxidation in an EC cell and correlation of the retention times obtained with the EC and MS detectors, information, such as antioxidant activity (oxidation potential), capacity (amount), and structural information, regarding individual antioxidants can be obtained. It is also shown that the antioxidant activity for some polyphenolic compounds and their oxidation products can be studied by varying the EC cell potential while monitoring the intensities for the compounds even for complex samples such as yellow onion extracts.

EXPERIMENTAL SECTION

Preparation of Solutions. Antioxidant stock solutions in methanol (MeOH, p.a., J.T. Baker, Deventer, The Netherlands)

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of quercetin dehydrate (Q, >98%, Fluka BioChemika, Buchs, Switzerland), kaempferol (K, >96%, Fluka BioChemika), resveratrol (R, Extrasynthese, Genay Cedex, France), and catechin (C, Extrasynthese), as well as quercetin-3-glucoside (Q3G, >90%, Fluka), quercetin-4′-glucoside (Q4′G, >97%, Polyphenols Laboratories AS, Sandnes, Norway), and quercetin-3,4′-diglucoside (Q3, 4′G, >97%, Polyphenols) were prepared and stored at −20 C. Antioxidant standard solutions in 10/90 (methanol/water, v/v %) were prepared daily from the stock solutions in 4 mL dark vials. The solutions were purged with nitrogen to remove oxygen.

The sodium formate buffer (pH 3.0) used in the off-line cyclic voltammetry measurements consisted of 200 mM NaHCOO/HCOOH (p.a., Merck KGaA, Darmstadt, Germany) in water. The antioxidant solutions used in the experiments were 1 mM solutions of antioxidant in 50% methanol and 50% buffer, also containing 0.5 M $NaNO_3$ (p.a., Merck) as supporting electrolyte. Ultrapure water (Milli Q, Millipore Corp., Marlborough, MA) was used for the preparation of all solutions. The pH was adjusted to 3.0 utilizing a pH meter (744 pH Meter, Metrohm AG, Herisau, Switzerland).

The ammonium formate buffer (pH 3.0) used in the on-line LC/EC/MS experiments contained 60 mM $NH_4HCOO/HCOOH$ (BDH Laboratory Supplies, Poole, England/Merck, Darmstadt, Germany) in water. In the isocratic LC experiments, the mobile phase was composed of 60% methanol and 40% buffer. Gradient LC experiments were performed with the same buffer−solvent system starting with 50/50 (buffer/MeOH) for 5 min, followed by an increase in the MeOH concentration to 90% during 5 min.

Preparation of Extracts. Yellow onion extract was prepared from chopped onion employing a previously described method[32] in which water is used as the extraction solvent at 120 C and 50 bar, and 3 × 5 min extraction time. The extracts were stored at −20 C and were freshly diluted 10 times with 10% methanol and purged with nitrogen prior to use.

Chemical Assay. The antioxidant capacity of the antioxidant standards was determined with a DPPH assay.[35] A stock solution (0.53 mg/mL) of DPPH was prepared daily with degassed methanol and then diluted 10 times to yield a working solution (53 μg/mL). Five different volumes of standard (7, 10, 30, 80, and 100 μL) were taken out to a 96-well microtiter plate and 200 μL of DPPH working solution was then added to each volume. In the final step, different volumes of degassed methanol were added to each well to give a total volume of 300 μL. The radical scavenging reaction with the antioxidants took place in the darkness during 4 h to make sure that the reaction was complete. The control sample (degassed methanol) was processed in the same way as the antioxidants.

Cyclic Voltammetry. The cyclic voltammetry (CV) measurements were performed in the absence of stirring using a MacLab system (model 4e, ADInstruments, Castle Hill, Australia) connected to a personal computer employing the PowerLab Echem and Chart4 software (ADInstruments). The working electrode was a 3 mm glassy carbon (GC) disk electrode (Bioanalytical Systems Inc., West Lafayette) while a platinum electrode was used as the counter electrode. The reference electrode was an Ag/AgCl electrode (model RE-6, Bioanalytical Systems Inc.). The GC electrode was frequently polished with Al_2O_3 (highly pure, KEBO Lab, Stockholm, Sweden) and subsequently cleaned by ultrasonication to remove residual abrasive particles. The experiments were made with scan rates of 10, 125, and 250 mV/s. The oxidation potentials of the antioxidant flavonoids are reported as peak potentials E_p versus the Ag/AgCl reference electrode unless stated otherwise.

Liquid Chromatography. Separations of quercetin, Q3G, Q4′G, and Q3,4′G were carried out at room temperature on an Agilent 1100 series LC system (G1312A, Walbronn, Germany) with a C_{18} column Gemini 3u 110 A; 3 μm; 150 mm × 2.00 mm (Phenomenex, Utrecht, The Netherlands) at a flow rate of 200 μL/min. The injection volume employed with the autosampler (Agilent 1100 G1367A) was 10 μL, and the vial tray was held at 4 C.

Mass Spectrometry. The mass spectrometry experiments were performed either with sample delivery by a syringe pump (Syringe Infusion Pump 22, Harvard apparatus Inc., Cambridge, MA) or by the LC system described above. Two different mass spectrometers were used, either an API *I* single quadrupole mass spectrometer (PE SCIEX, Concord, Canada) or the 3200 Linear Ion Trap Quadrupole LC/MS/MS Mass Spectrometer (3200 Q TRAP, Applied BioSystems Sciex Instruments, Foster City, CA), in the positive and negative ESI modes. Nitrogen, boil-off from liquid nitrogen without further purification, was used as curtain gas and nebulizer gas. For the API *I* instrument the following parameters were used, (negative ion mode in parentheses): ion spray voltage (ISV) 3800 V (−2200 V), interface plate voltage (IN) 650 V (−650 V), orifice lens voltage (OR) 50 V (−65 V), and ac entrance rod (R0) 30 V (−30 V). Q1 scans between m/z 10−1000 (step size 0.1), with a dwell time of 1.0 ms, count control (CC) 10, were recorded in the multichannel acquisition (MCA, summation of 10 scans, 25 scans in negative mode) mode after applying potential to the working electrode. The data were collected on a Macintosh computer by Tune 2.5-FPU software. The subsequent data analyses were performed with Analyst 1.4.2 software (Applied Biosystems) on a personal computer (Microsoft). For the 3200 Q TRAP instrument, several kinds of experiments were performed, with the Analyst 1.4.2 software (Applied Biosystems), including Q1 scan, SIM (selected ion monitoring), EMS (enhanced mass spectrometry), EPI (enhanced product ion), and IDA (information dependent acquisition).

On-Line EC/MS and LC/EC/MS. The EC/ESI-MS setup, including a modified electrochemical thin-layer flow cell (Bioanalytical Systems Inc.) coupled on-line to ESI-MS, has recently been described by Zettersten et al.[36] The EC flow cell was operated together with a battery-powered potentiostat (PalmSens, Palm Instruments BV, Houten, The Netherlands). To control the potentiostat, a Bluetooth wireless connected hp iPAQ Pocket PC (h 2200 series, Hewlett-Packard Company) was used along with the PalmScan software (vs 1.4.2, Palm Instruments BV). Although a stainless steel quasi reference electrode (QRE) was used in all the experiments, the potentials are reported versus an Ag/AgCl electrode to facilitate comparisons between the on-line EC/MS and off-line cyclic voltammetry experiments. The potential of the

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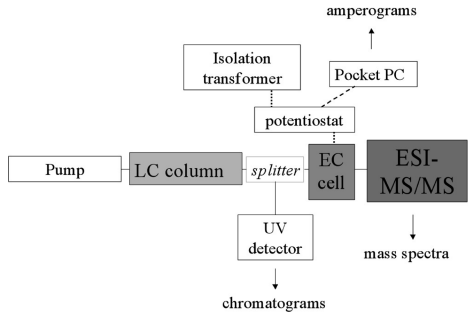

Figure 1. The employed on-line LC/EC/ESI-MS/MS system.

QRE was found to be +0.18 V with respect to the Ag/AgCl in the quercetin solution and this QRE potential was also assumed in the quercetin glucoside solutions. The length of the fused silica capillary (i.d., 50 μm; o.d., 185 μm) between the electrochemical cell and the API *I* MS was 0.4 m, yielding a transfer time of 9.4 s (at 5 μL/min) between the EC cell and the ESI-MS instrument. An isolation transformer PVM 500r (Tufvassons, Sigtuna, Sweden) was, unless stated otherwise, used to float the EC equipment and to avoid hazardous backward currents between the ESI emitter and the electrochemical cell and potentiostat.[37]

The on-line LC/EC/MS experiments were performed with a system schematically described in Figure 1. The LC system was coupled on-line to the EC cell and the ion trap MS. The LC pump delivered a flow rate of 200 μL/min which resulted in a transfer time of about 0.7 s between the EC and MS. Current vs time curves (amperograms) were recorded with the Pocket PC (Hewlett-Packard) employing the PalmTime software (vs 1.4.2, Palm Instruments BV). To avoid electric shocks, particular care should be taken when handling the floating LC/EC/ESI-MS system when the ESI high voltage is on.

RESULTS AND DISCUSSION

As is seen in Figure 2, depicting voltammograms for quercetin, kaempferol, catechin, and resveratrol, cyclic voltammetry was used for an initial characterization of the electrochemical behavior and determination of the oxidation potentials for the polyphenolic antioxidants studied in this work. Cyclic voltammetry have previously used to detect antioxidants[38] and to study antioxidant activity[29] although the technique is generally difficult to apply for the study of individual antioxidants present in complex samples. In Figure 2 it is seen that all four compounds were electroactive and that no corresponding reduction peaks were seen on the reverse scans indicating that the oxidation reactions were irreversible. As seen in Figure 2a, a clear oxidation peak was detected for quercetin at 0.43 V, which is in good agreement with previous reports[39,40] keeping in mind that the oxidation potential is pH dependent.[41] This relatively low peak potential demonstrates that quercetin is the most easily oxidized compound of the four compounds studied and the low oxidation potential indicates that quercetin should have a significant antioxidative activity. Kaempferol, which has a structure similar to that of quercetin and catechin, was found to exhibit one significant oxidation peak at 0.48 V (see Figure 2b) while two oxidation peaks at 0.46 and 0.81 V are seen in the voltammogram for catechin (Figure 2c). Two, albeit broad peaks, can be seen for resveratrol in Figure 2d. The first oxidation peak for resveratrol (at 0.57 V) has been ascribed to the oxidation of the phenol group.[42] From the shape of the voltammograms it is also clear that the initial oxidations of the compounds are followed by oxidations of the remaining hydroxyl groups at increasingly higher potentials. This means that the antioxidative capacity of these compounds should be dependent not only on the concentrations of the compounds but also on the redox potential at which the capacity is evaluated. On the basis of the oxidation peak potentials, quercetin can nevertheless be expected to be a slightly more active antioxidant than catechin, which in turn should be better than kaempferol and resveratrol. Since cyclic voltammetry cannot be used to detect individual antioxidants in a mixture an alternative approach is, however, needed for screening of antioxidants present in a real samples. As is described in the next sections, EC/ESI-MS is a much more appropriate method for this purpose particularly when combined with chromatography.

On-Line EC/ESI-MS. To investigate the possibilities of using EC/ESI-MS as a means to rapidly identify the presence of antioxidants in samples, experiments were performed with quercetin, kaempferol, catechin, and resveratrol. Infusion experiments, conducted at a flow rate of 5 μL/min, with the EC/ESI-MS system showed that the deprotonated species; quercetin, $[Q - H]^-$ (m/z 301), kaempferol, $[K - H]^-$ (m/z 285), catechin, $[C - H]^-$ (m/z 289), and resveratrol, $[R - H]^-$ (m/z 227) could be detected in the negative ESI-MS mode. All four compounds could likewise be detected in the positive ESI-MS mode as the following protonated species; quercetin, $[Q + H]^+$ (m/z 303), kaempferol, $[K + H]^+$ (m/z 287), catechin, $[C + H]^+$ (m/z 291), and resveratrol, $[R + H]^+$ (m/z 229), respectively. Upon oxidation of the compounds, new peaks in the mass spectra appeared which were not present when the EC cell was used under open circuit conditions. By application of different EC potentials while acquiring mass spectra, the oxidation potential of the four compounds could be determined and m/z for the oxidation products could also be determined in both the negative and positive ESI-MS modes (see Figure 3). As is seen in Figure 3 and Table 1, the oxidation of the four compounds generally resulted in the formation of several oxidation products, many of which underwent further oxidation as indicated by their decrease in intensities with increasing potentials. The latter finding, which is in good agreement with the results in Figure 2, is not unexpected as polyphenolic compounds can undergo several successive oxidations as a result of the fact that these compounds contain several electroactive hydroxyl groups. A complete description of the oxidation pathway of these compounds would therefore be difficult and time-consuming to attain. The identity of two products for quercetin detected in the positive mode, m/z 319 and 333, are, however, suggested to be due to the addition of −OH or CH_3O to an *ortho*-quinone in a similar way as proposed by

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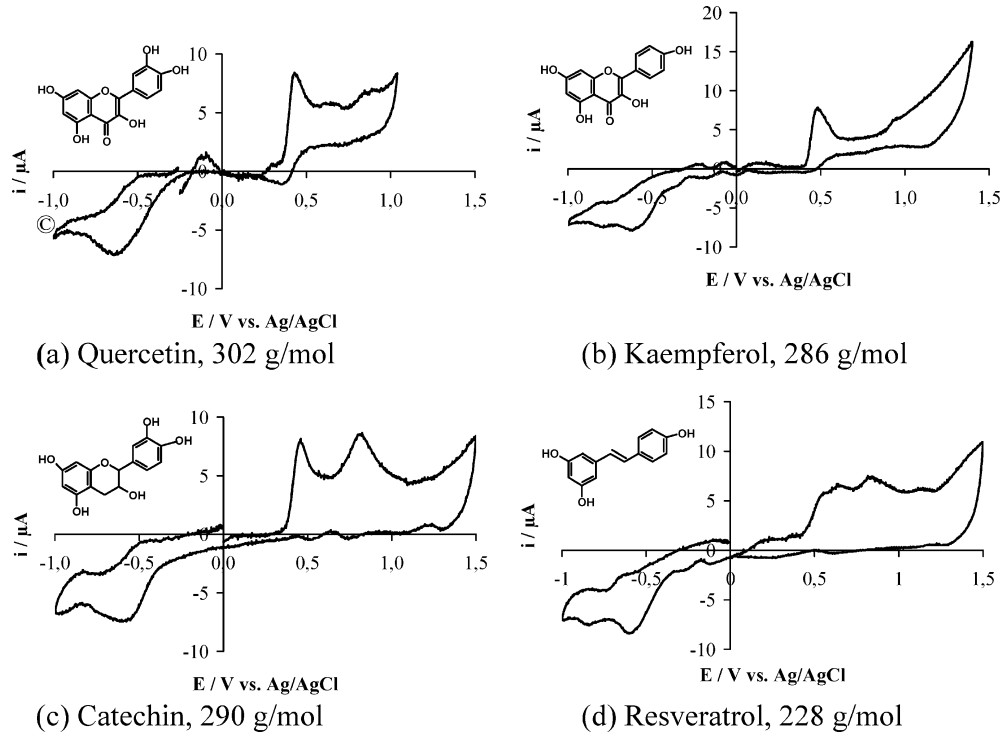

Figure 2. Cyclic voltammograms for 1 mM solutions of (a) quercetin, (b) kaempferol, (c) catechin, and (d) resveratrol recorded with a scan rate of 10 mV/s.

Zhou et al.[40] To simplify the identification of antioxidants in complex samples, the m/z values for the antioxidants and their oxidation products (see Table 1), as well as their dependence on the EC potential, can however be used, as will be further described below.

To estimate the antioxidant activity with the on-line flow system, the oxidation potentials for these compounds were determined based on the decrease in the intensity for the respective ions for quercetin, catechin, kaempferol, and resveratrol with increasing EC potential in both the positive and negative ESI-MS modes. In this way, half-wave potentials $E_{1/2}$ for the antioxidants could be obtained as is seen in Table 2. The half-wave potentials were generally taken as the averages of the values obtained in the positive and negative ESI-MS mode, respectively, as no statistically significant difference between these values were found (the variations in the $E_{1/2}$ values are most likely due to variations in the potential of the pseudo references electrode). It was then found that quercetin was the most active antioxidant (with the lowest oxidation potential) followed by catechin, kaempferol, and resveratrol. These findings are generally in good agreement with the off-line cyclic voltammetry results (see Table 2) indicating that the EC/ESI-MS system can indeed be used to both detect compounds with antioxidative properties and to determine their oxidation potentials.

On another mass spectrometer, a Qtrap instrument, similar on-line EC/ESI-MS results were obtained with a 50 μM solution of quercetin at a liquid flow rate of 200 μL/min in the positive ESI-MS mode. In these experiments, the intensities for a number of oxidation products for quercitin (i.e., m/z 319, 333, 336, 366, 380, 387, 648, and 680) were monitored as a function of the EC potential. It was found that the half-wave potential for quercetin species (m/z 303) was 0.25 V vs Ag/AgCl (which is in good agreement with the corresponding value obtained using Figure 3b) while the half-wave potential for the oxidation products at m/z 333 and m/z 387 species was about 0.2 V. The corresponding value for the m/z 380 species was about 0.8 V. These findings indicate that the m/z 333 and m/z 387 species (generated upon the oxidation of quercetin) were more easily oxidized than quercetin itself and that these species may have been oxidized to the m/z 380, m/z 366, and m/z 394 species when higher EC potentials were applied. This is also supported by the data for quercetin in Figure 3b in which it is seen that the m/z 333 species disappear when the potential was increased above about 0.5 V. For the m/z 319 and m/z 336 species, two plateaus with a first half-wave potential of 0.2 V and a second of 0.9 V were found. The intensities for the m/z 648 and m/z 680 species were also found to increase and decrease at about the same potentials yielding half-wave potentials for the latter species of 0.55 V. These results and the results in Figure 3 clearly show that the oxidation of quercetin, kaempferol, catechin, and resveratrol give rise to a range of oxidation products many of which are electroactive. This clearly shows that EC/ESI-MS significantly facilitates studies of the oxidation of polyphenolic compounds as it is possible to determine the potential dependence of the ion intensities for the oxidation products as a function of the potential. The results thus confirm the results obtained in a previous study[40] that the oxidation pathway for these polyphenolic compounds is quite complex involving a mixture of different oxidation products.

On-Line LC/EC/ESI-MS. In the analysis of real samples containing a multitude of antioxidants, a separation of the compounds prior to the EC/ESI-MS system is generally desirable to more easily obtain the desired information about the individual antioxidants. To challenge the developed on-line system, experiments were therefore also carried out using a biological sample, in this case, an onion extract. Since the most abundant species in onion are quercetin and its glycosides,[32] tests with solutions

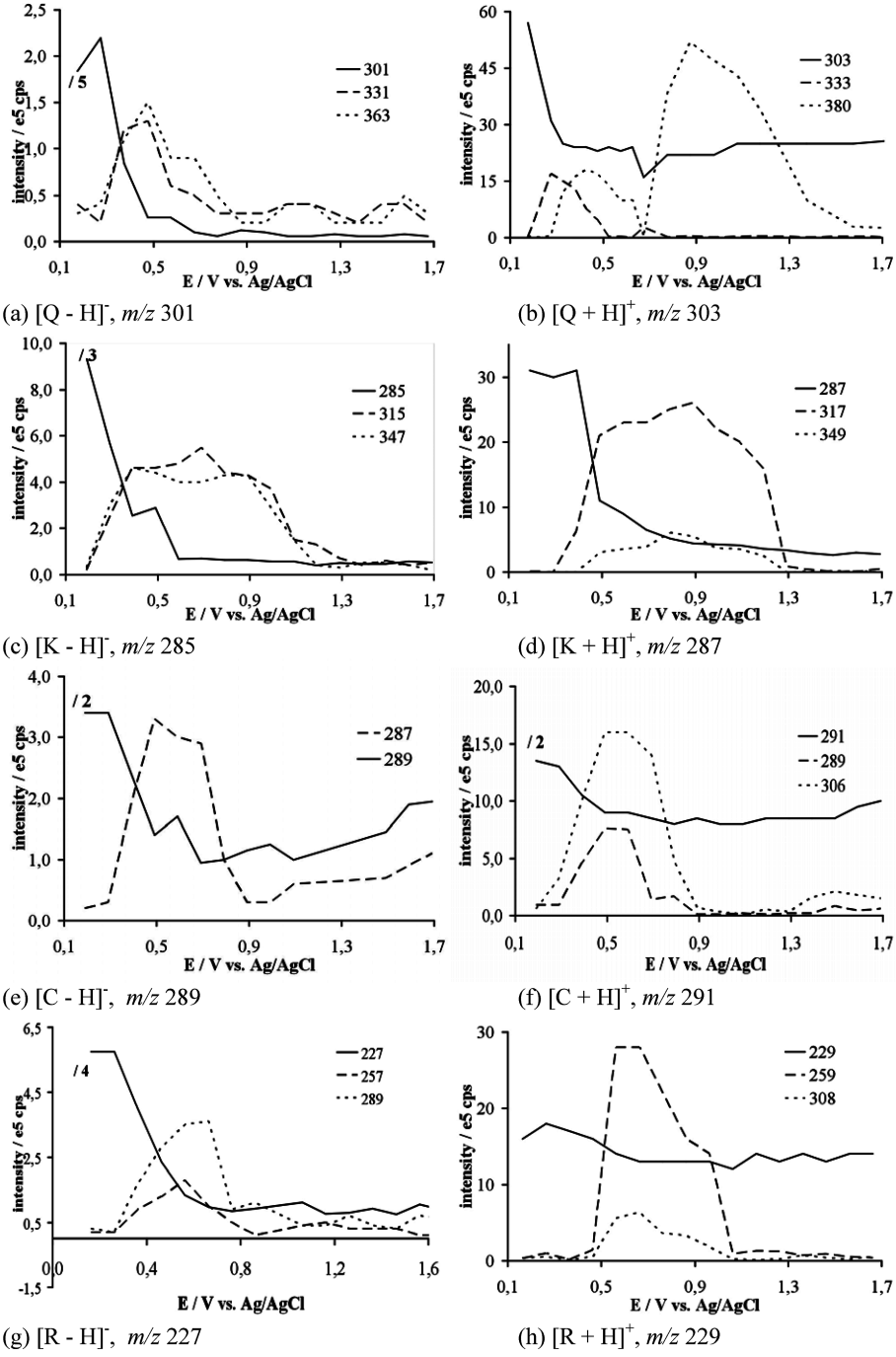

Figure 3. Mass spectral intensity as a function of applied electrochemical potential *E* (vs Ag/AgCl), in negative (a, c, e, g), and positive (b, d, f, h) ESI-MS (API *I*) for 5 μM of each analyte. The *m*/*z* for major oxidation products are given in the legends of the graphs. The flow rate was 5 μL/min.

containing quercetin (Q), quercetin-3-glucoside (Q3G), quercetin-4′-glucoside (Q4′G), and quercetin-3,4′-glucoside (Q3,4′G) were, however, performed prior to the analysis of the real sample. It would of course also be possible to perform the LC separation after the EC cell as was shown by Lohmann et al.[43] Although the latter setup is better suited for the identification of the oxidation products, a separation before the EC cell is more appropriate when screening for polyphenolic antioxidants in a sample.

As is seen in Figure 4a, b, which show the EC current as a function of time at a potential of 0.6 and 1.6 V, respectively, employing a solution containing equal concentrations (i.e., 10 μM) of Q, Q3G, Q4′G, and Q3,4′G, all four compounds were electroactive and could be separated on the LC column. In the figure, it is also seen that the elution order was as follows: (1) Q3,4′G, (2) Q3G, (3) Q4′G, and (4) Q. By comparison of the results in parts a and b of Figure 4, it is clear that a higher oxidation potential favors the detection of the diglucoside (Q3,4′G). This is more clearly seen in Figure 4c, showing hydrodynamic voltammograms for the same four compounds. It is seen that although a higher potential was needed to oxidize the glucosides than quercetin itself (see also Table 3), the oxidation currents for the glucosides increased significantly faster than that for quercetin when the

(43) Lohmann, W.; Karst, U. *Anal. Chem.* **2007**, *79*, 6831–6839.

Table 1. *m/z* for Oxidation Products Generated with 5 μM Quercetin, Kaempferol, Catechin, and Resveratrol in the Positive and Negative ESI Modes As Depicted in Figure 3

compound	ion mode	monitored ions[a] *m/z*
quercetin	neg	**301**, 329, 347, 361, 363
	pos	**303**, 319, 333, 336, 366, 380, 387, 648, 680
kaempferol	neg	**285**, 301, 315, 333, 347
	pos	257, 285, **287**, 303, 317, 320, 331, 335, 349, 352, 357, 366, 371, 380, 387
catechin	neg	287, **289**
	pos	289, **291**, 306, 311
resveratrol	neg	**227**, 243, 257, 275, 287, 289
	pos	**229**, 245, 259 308, 313

[a] The *m/z* in bold corresponds to the protonated or deprotonated ion from the initial compound.

Table 2. Oxidation Potentials for the Antioxidants Determined by Cyclic Voltammetry and EC/ESI-MS

antioxidant	off-line CV: E_p/V (vs Ag/AgCl)	on-line EC/ESI-MS: $E_{1/2}$/V (vs Ag/AgCl), $n = 2$[a]
quercetin	0.43	0.40
catechin	0.46	0.44
kaempferol	0.48	0.49
resveratrol	0.57	0.53

[a] Number of measurements.

potential was increased. This can be explained by the larger number of oxidizable hydroxyl groups present on the molecule for the glucosides and the fact that increasingly higher potentials are needed to oxidize the remaining hydroxyl groups. The latter explains the rapid increase in the current for the diglucoside Q3,4′G for potentials above approximately 0.7 V. These results show that the antioxidant capacity of a sample containing these polyphenolic species will depend on at which redox potential (i.e., with which chemical assay) the determination is carried out. A sample containing equal amounts of quercetin and quercetin-3,4′-glucoside (Q34′G) will thus have completely different antioxidant capacities at, e.g., 0.4 and 0.7 V. A significant advantage of the present LC/EC/ESI-MS approach is consequently the possibility to study the antioxidant activity and capacity at any potential desired. By application of different oxidation potentials to the EC cell, the selectivity can thus be altered in such a way that the detected EC peak area and the ion intensity in the MS, both of which are related to the concentration and thus the capacity of the antioxidants, can be evaluated as a function of the EC potential. Previous reports regarding antioxidant capacity[10,14,18,19] have shown that results obtained from EC measurements can show some correlation with results obtained with chemical assays but so far it has generally been difficult to establish which EC potential that should be used in such comparisons. In this work, the antioxidant capacity for Q, Q3G, Q4′G, and Q3,4′G were also determined using the DPPH assay.[35] It was then found that Q had the highest antioxidant capacity followed by Q3G, Q4′Q, and Q3,4′G. As is seen in Figure 4c, Q exhibited the largest EC current at an EC potential of 0.4 V indicating that this compound had the highest antioxidant capacity at this potential while Q3,4′G exhibited the highest capacity at potentials above 1.6 V (note the different current scales for Q and the other compounds in Figure 4c). These results thus indicate that the redox potential in the DPPH experiments was in the vicinity of 0.4 V. As mentioned above, these findings in Figure 4c can be explained by the fact that Q is the most easily oxidized compound while Q3,4′G has

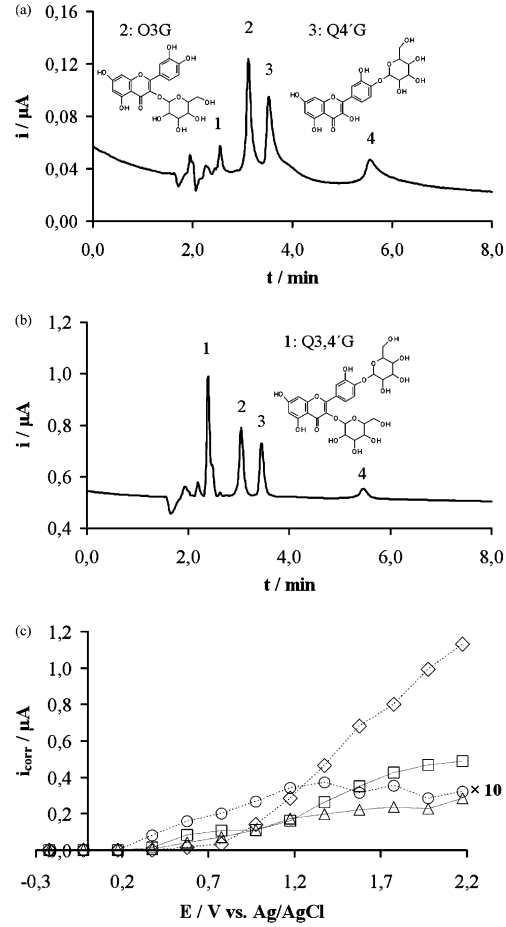

Figure 4. LC/EC (a) amperogram obtained at 0.6 V vs Ag/AgCl (b) amperogram, obtained at 1.6 V vs Ag/AgCl (c). Baseline corrected hydrodynamic voltammograms for 10 μM each of Q (○), Q3G (□), Q4′G (△), and Q3,4′G (◇) in 10% methanol using a flow rate of 200 μL/min and isocratic elution (40% ammonium formate buffer and 60% methanol).

Table 3. LC/EC/ESI-MS of 10 μM Q, Q3G, Q4′G, and Q3,4′G in 10% Methanol with Intact Protonated Analyte Ions in Bold

peak	*m/z*	identity	$E_{1/2}$/V vs Ag/AgCl
1	**627**	**[Q3,4′G + H]$^+$**	0.67
	465	[QG + H]$^+$	0.70
	303	[Q + H]$^+$	0.70
	366		0.55
	380		0.55
	679		0.75
	695		0.70
2	**465**	**[Q3G + H]$^+$**	0.35
	303	**[Q + H]$^+$**	0.40
	380		0.60
3	465	**[Q4′G + H]$^+$**	0.35
	366		1.00
	380		0.80
4	303	[Q + H]$^+$	0.40
	380		0.70

Table 4. Fragmentation of Quercetin and Its Glucosides (10 μM Each) in the Positive and Negative ESI Modes

ion mode	peak	parention m/z	parent ion identity	fragment ions m/z
neg	1	625	$[Q3,4'G - H]^-$	463, 300, 271, 179, 151
pos	1	627	$[Q3,4'G + H]^+$	465, 303
neg	2	463	$[Q3G - H]^-$	300, 271, 255, 243, 227, 151
pos	2	465	$[Q3G + H]^+$	303, 229, 137
neg	3	463	$[Q4'G - H]^-$	301, 179, 151, 121, 107
pos	3	465	$[Q4'G + H]^+$	303
neg	4	301	$[Q - H]^-$	273, 179, 151, 107
pos	4	303	$[Q + H]^+$	285, 257, 247, 229, 201, 173, 163, 153, 137

many more oxidizable hydroxyl groups which contribute to the capacity at sufficiently high potentials (see the molecular-structures inset in Figure 4). This indicates that direct comparisons of the results obtained with electrochemistry and different chemical assays generally are difficult since the relevant redox potentials of the different assays and the influence of kinetic effects need to be known. It should also be pointed out that chemical assays, such as the DPPH assay, generally assess the total capacity of the sample on a much longer time-scale compared to that used in the present LC/EC/ESI-MS system or in other techniques based on, e.g., cyclic voltammetry. In the present system, a complete oxidation of the compounds was not obtained as a result of the limited residence time in the EC flow cell (the conversion efficiency of the present EC cell was about 50%). The proposed system is, on the other hand, well-suited for rapid screenings of antioxidants in complex samples during which individual compounds with desired oxidation potentials are to be rapidly identified.

In the LC/EC/ESI-MS experiments, the retention times for quercetin and its glucosides obtained with the EC detector were also compared with the retention times obtained with the MS detector. This approach greatly facilitates the identification of the electroactive species with mass spectrometry. The results from the EC cell can then be used to detect electroactive species whose identity and oxidation products then can be further studied in the mass spectrometer. The MS spectra for Q, Q3G, Q4′G, and Q3,4′G were recorded both in the negative and positive ionization mode and peaks corresponding to protonated or deprotonated molecules were identified for all four compounds (see Table 4). Because of up-front fragmentation, a loss of sugar (neutral loss of 162 u) could also be seen for the glucosides. Upon an increase in the oxidation potential (from −0.2 to 2.2 V in steps of 0.2 V), the appearance of the mass spectra changed slightly as new peaks, most probably corresponding to oxidation products, were seen. A list of some of the observed peaks for the four compounds and the half-wave potentials for some generated products can be found in Table 3. The species corresponding to m/z 366, m/z 380, m/z 679, and m/z 695 were most probably generated by electrochemical oxidation and may originate from oxidation products of quercetin (and its glucosides). The most dominant oxidation product was the m/z 380 compound. To gain additional information about the molecular structure of the electroactive species detected by the LC/EC/ESI-MS system one would need to carry out additional experiments with tandem mass spectrometry (MS/MS). Before

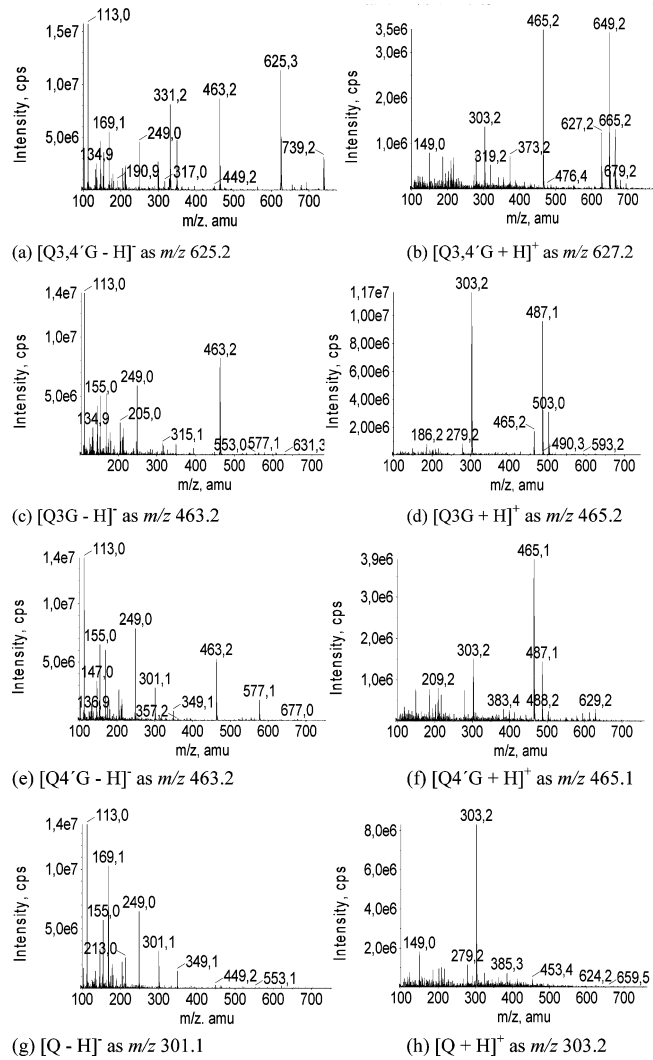

Figure 5. Enhanced mass spectra for 10 μM of each compound in 10% methanol with negative (a, c, e, g) and positive (b, d, f, h) ESI (ion trap MS) for an EC potential of 1.4 V vs Ag/AgCl. Gradient elution at a flow rate of 200 μL/min was employed.

presentation of MS/MS results, there are some additional experimental parameters that deserve attention.

Variation of the Volumetric Flow Rate. In EC/ESI-MS involving thin layer electrochemical flow cells, it is well-known[36,37] that the conversion efficiency of the cell increases with decreasing flow rate. In a LC/EC/ESI-MS system, the use of low flow rates, however, give rise to long analysis times and significant band broadening of the peaks. It is therefore important to investigate the influence of different flow rates on the EC and MS detection of the oxidation products in the present system. An additional advantage of placing the EC cell after the LC column was that the separations then always could be performed with a flow rate of 200 μL/min even though the flow rate through the EC cell and the ESI emitter was varied using different split ratios after the LC column. For a reduced flow rate through the EC cell (6 and 31 μL/min), the peaks in the EC as well as in the MS chromatograms became broader and smaller due to the increased time for diffusion of the sample plug. The transfer time between the EC cell and the MS also increases with decreasing flow rate, which could be a problem for unstable oxidation products. At flow rates of 66 and 200 μL/min, the peaks in the chromatograms were,

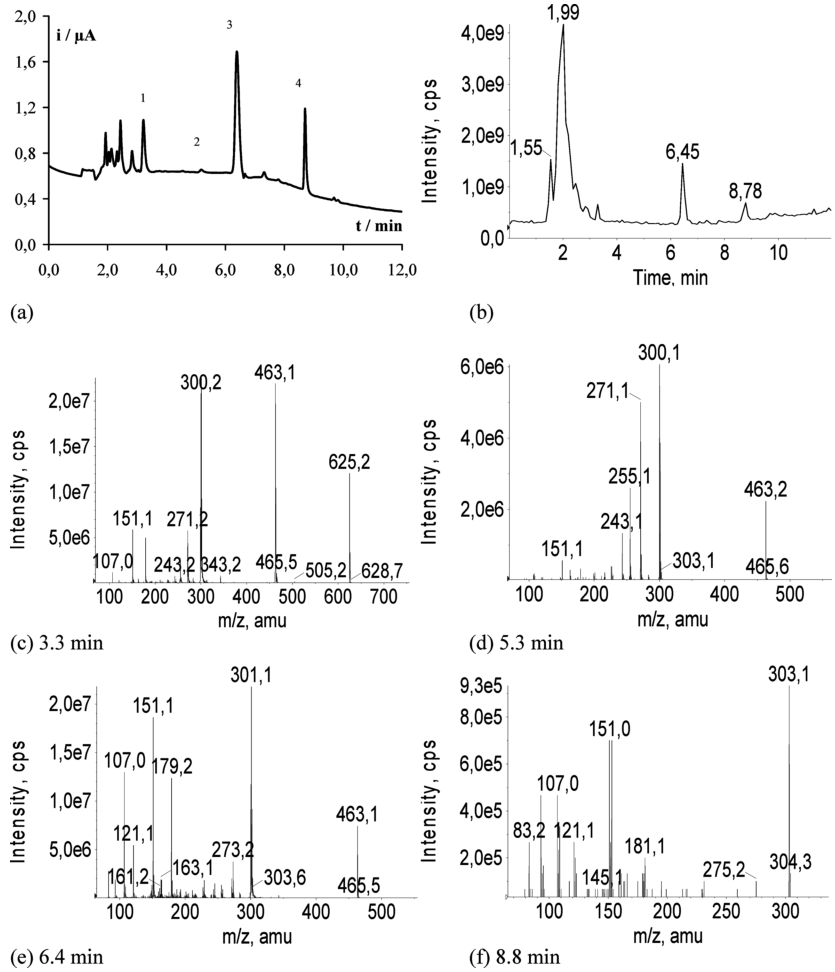

Figure 6. LC/EC/ESI-MS/MS of yellow onion extract as an (a) amperogram, (b) total ion chromatogram (TIC), and MS/MS spectra from enhanced product ion (EPI) scans for deprotonated (c) Q3,4′G as *m*/*z* 625, (d) Q3G as *m*/*z* 463, (e) Q4′G as *m*/*z* 463, and (f) Q as *m*/*z* 301 for an EC potential of 1.4 V vs Ag/AgCl. Gradient elution at a flow rate of 200 µL/min was employed.

as expected, more distinct, sharper, and symmetric (due to less band broadening). It was also found that oxidation products could be detected even at the highest flow rate used (i.e., 200 µL/min) indicating that the present system was compatible, at least, with volumetric flow rates of between 6 and 200 µL/min. This is important as this means that it would be possible to study the stability of generated oxidation products by varying the transfer time. At a flow rate of 200 µL/min, the transfer time between the EC cell and the MS is only approximately 0.7 s (which should be compared with 23 s at a flow rate of 6 µL/min). This possibility to employ higher flow rates should facilitate the detection of relatively unstable oxidation products.

Interferences Due to the Presence of a Ground Point. As has been discussed previously,[36,44,45] it is important to decouple the electrochemical cell from the high voltage of the mass spectrometer for example by floating the potentiostat and the EC cell on the ESI high voltage employing an isolation transformer. The introduction of a ground point between the EC cell and the MS is not recommended although ground points are frequently used in LC/ESI-MS systems for safety reasons. In the present work, interferences as a result of the presence of a ground point between the EC cell and the ESI emitter were clearly seen in the negative ESI mode as oxidation products of quercetin (at *m*/*z* 331, 361, 364, 385) could be detected even for potentials at which no oxidation took place in the EC cell. This generation of the oxidation products can be explained by the fact that an oxidation reaction took place at the ground point as a result of a backward current between the ESI emitter and the ground point.

LC/EC/ESI-MS/MS. To increase the power of mass spectrometric detection of quercetin and some of its glucosides in combination with LC separations, an information-dependent acquisition (IDA) was performed in both the positive and negative ion mode. A survey scan (EMS) was first recorded followed by a selection of the two strongest *m*/*z* signals for a further MS/MS scan (EPI). In Figure 5, enhanced mass spectra (EMS) for 10 µM each of Q, Q3G, Q4′G, and Q3,4′G, are shown for negative (a, c, e, and g) as well as positive (b, d, f, and h) ion mode for an applied potential of 1.4 V vs Ag/AgCl. In Table 4, parent ions of quercetin and its glucosides as well as the major fragments of the parent ions are listed. Some sodium and potassium adducts for the different compounds were also detected. The data in Table 4 (and similar data for other compounds) can clearly be used to facilitate the identification of compounds which undergo oxidations

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(45) Kertesz, V.; Van Berkel, G. J.; Granger, M. C. *Anal. Chem.* **2005**, *77*, 4366–4373.

at a specific potential. In the next section, this is also demonstrated for a sample containing a range of different compounds.

LC/EC/ESI-MS/MS of Yellow Onion Extract. When a more complex mixture is to be separated, gradient elution is generally needed, which puts higher demand on the detection system since changes in the composition of the mobile phase may give rise to drift in the background levels. To test the present LC/EC/ESI-MS/MS system with respect to a separation of a complex sample employing gradient elution conditions, a yellow onion (*allium cepa*) extract was analyzed. As is seen in Figure 6a, showing the EC chromatogram, this sample contained a number of species that underwent oxidation at a potential of 1.4 V. Despite the slightly sloping baseline, it is clear that the EC cell is compatible also with this type of gradient elution. As is seen in Figure 6b, peaks were also seen in the TIC (total ion chromatogram) where the broad peak in the beginning of the chromatogram was due to numerous coeluting compounds. By relation of the retention times obtained with the EC and MS detectors, it was possible to identify Q3,4′G (1), Q3G (2), Q4′G (3), and Q (4) in the onion extract with the help of the previously described information regarding the m/z values for the oxidation products. This finding is in good agreement with the findings of Zielińska et al.[38] who reported that the dominant forms of quercetin in onion included Q3,4′G (47.8%), Q4′G (43.4%), Q3G (5.0%), as well as free quercetin (2.9%), based on the use of a combination of hydrodynamic and cyclic voltammetry, LC with amperometric detection, and LC/UV/ESI-MS. In the present study, high-quality MS/MS spectra were likewise possible to obtain during an IDA experiment on a complex sample as shown in Figure 6c−f. Acquiring MS/MS spectra for the oxidation products was, however, found to be more challenging, generally due to the low intensity signals, which require careful adjustments of the IDA parameters prior to analysis. The fact that quercetin and its three glucosides straightforwardly could be detected in the yellow onion extract using the present LC/EC/ESI-MS/MS method clearly shows that this experimental approach should be a valuable complement to the total capacity chemical assays commonly used in the studies of antioxidants such as the polyphenols used in this work.

CONCLUSIONS

It has been shown that an electrochemical cell coupled to liquid chromatography and electrospray ionization mass spectrometry can be used to rapidly detect and identify antioxidants, such as quercetin and some different quercetin glucosides in complex samples. As has been exemplified using catechin, kaempferol, and resveratrol, the LC/EC/ESI-MS/MS system will most probably work for a range of other polyphenolic compounds. It is also reasonable to assume that the approach can be used for many other electrochemically active compounds. The LC/EC/ESI-MS/MS system enables rapid screening for compounds undergoing oxidation in complex samples as well as the possibility to simultaneously determine the oxidation potentials (antioxidant activities) for a range of compounds present in complex samples. Unlike the chemical assays commonly used for the determination of the total antioxidant capacity of a sample, the present LC/EC/ESI-MS/MS system thus provides information about the chemical structures of individual compounds in the samples as well as their antioxidative activities and capacities. This approach should therefore be a valuable complement to time-consuming chemical assays, which focus on the total antioxidative capacity of a sample rather than the properties of the individual antioxidants.

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