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ORIGINAL PAPER

Study of electrochemical oxidation of cyanidin glycosides by online combination of electrochemistry with electrospray ionization tandem mass spectrometry

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Abstract Electrochemistry coupled with mass spectrometry (EC–MS) is a promising analytical tool for the online study of oxidation processes of anthocyanins. Two cyanidin glycosides, cyanidin-3-galactoside (ideain) and cyanidin-3,5-diglucoside (cyanin), were subjected to electrochemical oxidation and subsequent online mass spectrometric identification of the formed products. Application of relatively low working potentials (around 100 mV vs. Pd/H_2) using a porous graphite coulometric electrode yielded detectable oxidation products. As determined by hydrodynamic voltammetry, the monoglycosylated analogue undergoes anodic oxidation easier than the diglycosylated one. As a first step of the electrochemically induced oxidation, incorporation of a hydroxyl group was observed for both glycosides. Besides, an oxidative condensation of two anthocyanin molecules was observed. The proposed oxidative condensation was further confirmed by consecutive fragmentation (i.e., collection of MS^2, MS^3, and MS^4 spectra) in which corresponding subsequent losses of the sugar moiety were observed.

Keywords Cyanidin · Anthocyanin · Electrochemistry · Oxidations · Mass spectrometry

Introduction

The first serious attempts to combine electrochemistry (EC) with mass spectrometry (MS) date back to the early 1970s. Nevertheless, these pioneering works stayed rather aside from the main analytical stream over the next several decades. The interest in this hyphenated technique, however, has been rapidly growing in recent years; this is even more evident by the continuously increasing number of scientific papers dealing with this topic [1]. The expansion of electrospray ionization (ESI–MS) as a soft ionization technique in recent years also plays a major role in the current EC–MS development [2]. ESI is an ionization technique which is well compatible with electrochemistry. The EC–MS technique (often in combination with previous separation by liquid chromatography) can be particularly useful in the case that separation is required for the study of electrochemical properties of an individual compound in a mixture and/or separation is required for electrochemical conversion product(s). Electrochemical cells that are easily coupled to ESI–MS instruments became commercially available within recent years. Porous graphite coulometric working electrodes, combined with the maintenance-free palladium–hydrogen reference electrode, are among the most popular systems. At their large electrode surface, high conversion at moderate flow rates of the mobile phase can be achieved (<0.1 cm^3/min).

Current applications of the EC–MS technique can be divided into four main areas [3]: (i) online electrochemical derivatization prior to MS; (ii) specific protein/peptide cleavage [4, 5]; (iii) studies of electrochemical reaction mechanisms [6, 7], including studies of short-lived intermediates [8]; (iv) in vitro mimicking of biological processes [9–11], approaching metabolic oxidation [12, 13]. Such EC–MS applications are a very promising starting point for further metabolic studies and drug metabolism mimicking in living organisms.

Electrospray MS (ESI–MS), thanks to its demands on mobile phase composition and flow rates, offers very good EC compatibility, although it has to be taken into account

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that the ESI outlet capillary also itself acts as a current-controlled electrochemical cell and thus diverse further electrochemical reactions can occur [14, 15].

As coulometric detection yields up to 100% conversion, coulometric cells can be used as online flow-through reactors, enabling quantitative transformation of electroactive reactants into their oxidized (or reduced) forms.

Anthocyanins are natural water-soluble dyes that are responsible for the red, blue, and purple colors of numerous flowers and fruits. Anthocyanins, as glycosylated derivatives of the 3,3′,4′,5,7-pentahydroxyflavylium cation, are generally classified as flavonoids. The electron deficiency of the flavylium cation makes the free aglycons (anthocyanidins) highly reactive and much less stable than the glycosides. Consequently the aglycons occur much less frequently in nature. Up to now, over 500 different anthocyanins have been isolated from plant material [16].

Anthocyanins have several health-promoting effects, notably antimicrobial and antiviral properties. Beside, formulations rich in anthocyanins were used for the prevention and treatment of diabetes, cardiovascular diseases, some forms of cancer and obesity [17–19]. Anthocyanins are also natural antioxidants that are attracting attention because of their chemoprotective role in the oxidative metabolism of cells.

There are several articles dealing with the use of high-performance liquid chromatography (HPLC) coupled with electrochemical detection for the analysis of anthocyanins (e.g., [20, 21]). The obtained data served as supporting information for the MS-based identification of anthocyanins in flavonoid-rich samples. Moncada et al. [22] reported an interesting electrochemical oxidation of kuromanin (cyanidin-3-glucoside) and a synthetic analogue bearing no hydroxyl on the B ring of the anthocyanidin skeleton. These authors (following the previous study of catechols [23]) proposed the formation of quinones followed by a reaction with an original molecule to form a dimeric structure. Cyanidin glycosides (for their structure, see Fig. 1) belong to the group of the most frequently occurring anthocyanins and, therefore, they extensively contribute to the human diet.

In this paper, we report the use of EC–MS to investigate processes involved in the oxidation of anthocyanins. To the best of our knowledge, there is no previous study of anthocyanins using online coupling of electrochemical transformation with ESI–MS. The observed electrochemical conversion contributes to our understanding of the transformation processes of anthocyanins in biosystems.

Results and discussion

As can be seen from the hydrodynamic voltammograms in Fig. 2, anthocyanins can be easily oxidized electrochemically.

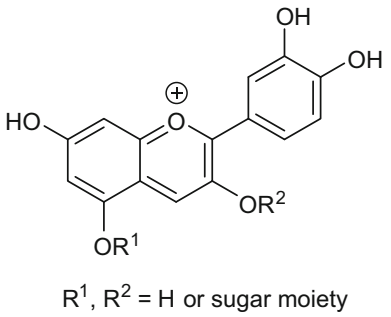

Fig. 1 Structure of cyanidin glycosides

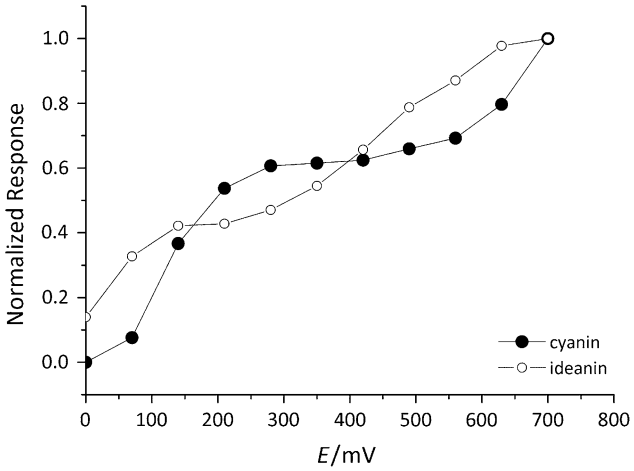

Fig. 2 Hydrodynamic voltammograms of cyanidin-3,5-diglucoside (cyanin) and cyanidin-3-galactoside (ideain) (normalized response expressed as a relative peak area A; $A_{max} = 1$)

The monoglycosylated analogue is oxidized at even lower potentials than the diglycosylated one. When an appropriate electrochemical cell is coupled with the mass spectrometer the oxidation products can be studied online. Relatively low potentials (around 100 mV vs. Pd/H_2, see Fig. 3) allow the formation of detectable oxidation products.

Figure 4 shows the mass spectra obtained when cyanidin-3-galactoside is passed through the electrochemical cell. The upper spectrum is obtained when the electrochemical cell is switched off and corresponds to the spectrum obtained using direct injection of a standard into the ion source. The dominant ion corresponds to the flavylium cation of the original dye (i.e., m/z 449, $[M]^+$). Minor peaks at m/z 471 and 919 correspond to adducts with sodium (i.e., $[M - H + Na]^+$ and $[2M - 2H + Na]^+$, respectively). The peak at m/z 897 can be ascribed to a monocharged dimer formed from the original dye (i.e., $[2M - H]^+$). Those ions are commonly formed during the ESI process. The bottom spectrum shows the situation when the potential +100 mV vs. $Pd/H_2$is applied on the electrochemical cell. Besides the molecular ion of cyanidin-3-galactoside (m/z 449) a peak at m/z 465 appeared.

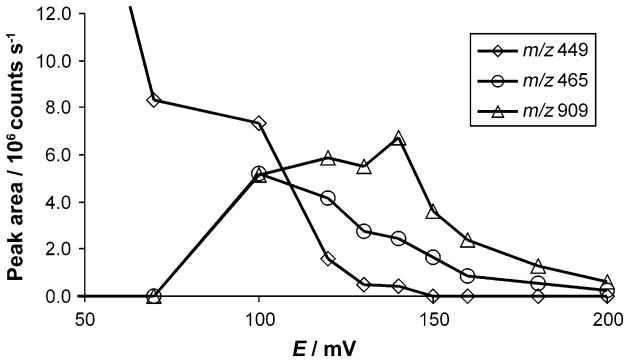

Fig. 3 Effect of applied voltage on the response of cyanidin-3-galactoside and arising oxidation products measured by MS (peak area obtained by integration of peak of eluting compound in an ion chromatogram reconstructed at related *m/z* value)

The difference of both values corresponds to 16 mass units, suggesting the introduction of one oxygen atom into the anthocyanin molecule. This process can be very likely explained as hydroxylation. A tandem mass spectrometer allows one to isolate the formed ions and to study their fragmentation after collision-induced dissociation in the ion trap. Figure 5a shows the fragmentation of the oxidation product *m/z* 465. The loss of water (formation of ion at *m/z* 447) can be observed. As a dominant process, however, loss of the sugar moiety occurs (Δ*m/z* 162) and the related aglycon is formed (*m/z* 303; isomer of delphinidin). The loss of the sugar unit corresponds to the main fragmentation pattern of the original pigment (i.e., the fragment at *m/z* 287 dominates: 287 = 449 – 162). The spectrum, therefore, supports the identification and proves that the oxidation occurs on the anthocyanidin skeleton (the sugar moiety remains unchanged during the "soft" oxidation).

Further fragmentation of the aglycon (although providing a weak signal) leads to the typical cascade of neutral losses of water (Δ*m/z* 18) and carbon monoxide (Δ*m/z* 28) (Fig. 5b).

The main fragments obtained after collision-induced dissociation of the aglycon correspond to the fragments found in the MS^2 spectrum of delphinidin [24]. However, as a result of the weak signals obtained in the MS^3 spectrum and the lack of isomer standards the position of hydroxylation was not satisfactorily located. This is an objective of future experiments. Besides, an ion at *m/z* 909 was detected after oxidation (Fig. 4) and in the collision spectra two consequent losses of galactose units are distinctly observed (Fig. 6). This can be explained as an oxidative condensation of two ideain molecules.

As mentioned in the "Introduction", a couple of previous articles also suggest an oxidation and condensation of catechol-bearing compounds [23]. Our mass spectrometric data therefore support this idea. During electrochemical transformation a peak at *m/z* 701 is also observed with a lower intensity compared with those of the oxidation products discussed above (Fig. 4). The corresponding structure, however, has not been elucidated yet.

Similar processes were observed when a diglycosylated analogue (i.e., cyanidin-3,5-diglucoside, cyanin) was electrochemically oxidized (Fig. 7).

The ion at *m/z* 627 dominates in the bottom spectrum (*E* = 140 mV vs. Pd/H_2electrode) corresponding to monohydroxylation of cyanidin-3,5-diglucoside. The ion at *m/z* 465 represents an oxidized cyanidin-monoglucoside arising due to elimination of the sugar moiety from the ion at *m/z* 627 in the ion source. The minor ion at *m/z* 649 corresponds to the mass of the sodium adduct of the oxidized

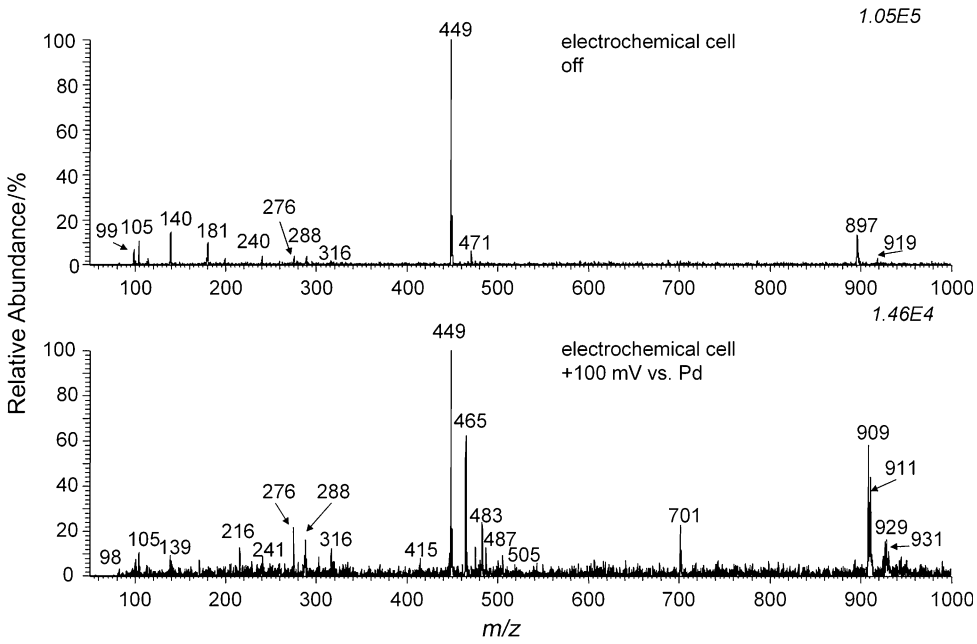

Fig. 4 MS spectra of cyanidin-3-galactoside (*top*) and products of its oxidation at +100 mV (vs. Pd/H_2) (*bottom*)

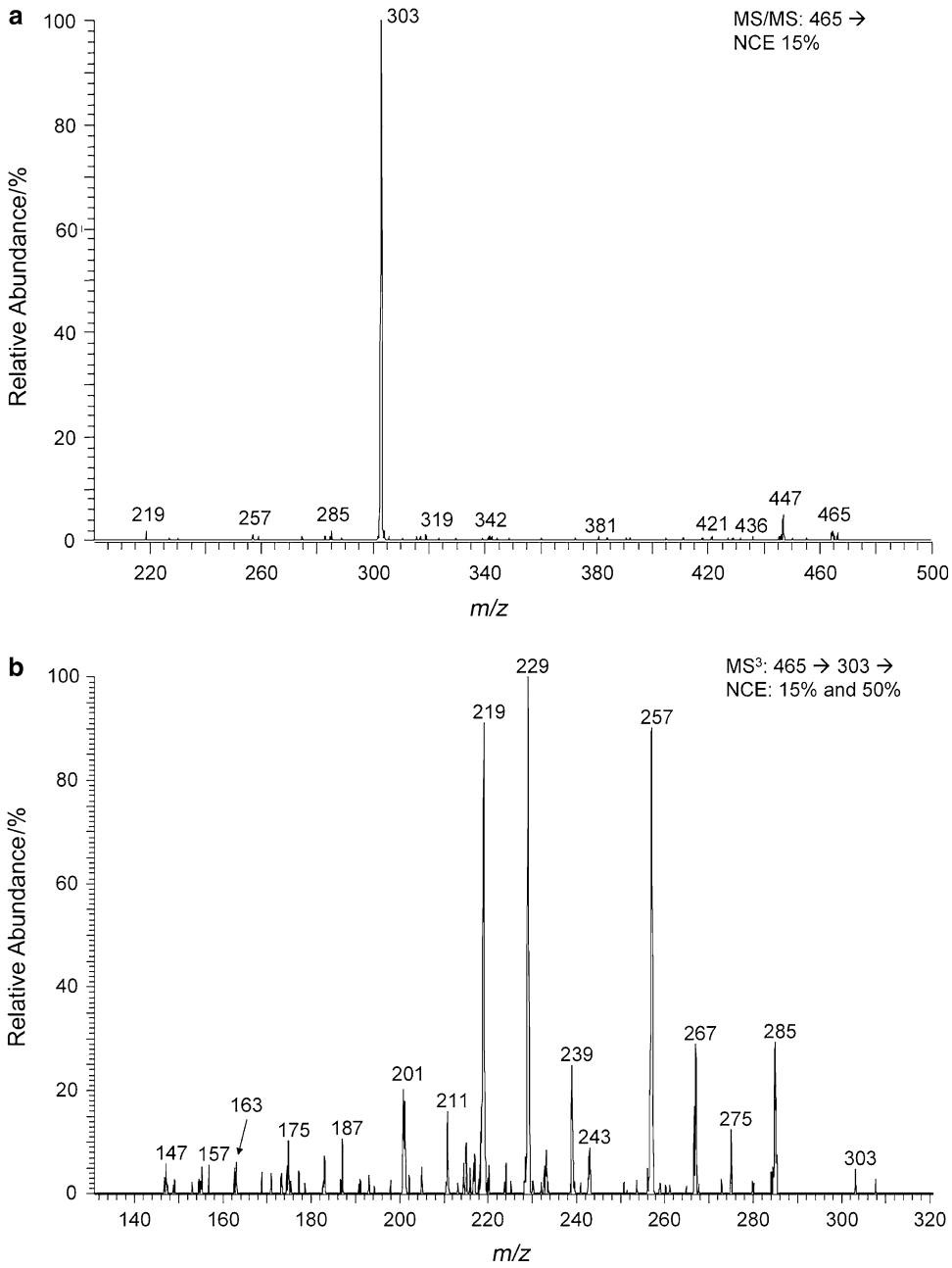

Fig. 5 **a** MS^2 spectrum of the oxidized product of cyanidin-3-galactoside at *m/z* 465. **b** MS^3 spectrum of the aglycon arising from fragmentation of the oxidized product at *m/z* 465

compound $[M - H + Na]^+$. Formation of sodium adducts is, as has already been mentioned, a typical process accompanying ESI and even trace amounts of sodium ions can result in the appearance of these adducts. More interesting is the formation of an ion at *m/z* 1,233 (inset in Fig. 7). Four sequential losses of glucose units are observed in the MS^2 of this ion (Fig. 8), and related MS^3 and MS^4 spectra confirmed that these cleavages are consecutive (data not shown).

The fragmentation pattern supports the idea of oxidative condensation of anthocyanins. Although generally many possible structures can be taken into account for oxidative condensation of anthocyanins, in the case of the diglycosylated derivative some processes are blocked (the two hydroxy groups in positions 3 and 5 are glycosylated). Thus the number of potential reaction sites is decreased. Two speculative structures resulting from a condensation on the C or A ring of the anthocyanidin skeleton, respectively, are shown in Fig. 9.

Experimental

Chemicals

Standards of anthocyanins (cyanidin-3-galactoside chloride and cyanidin-3,5-diglucoside chloride) were obtained from

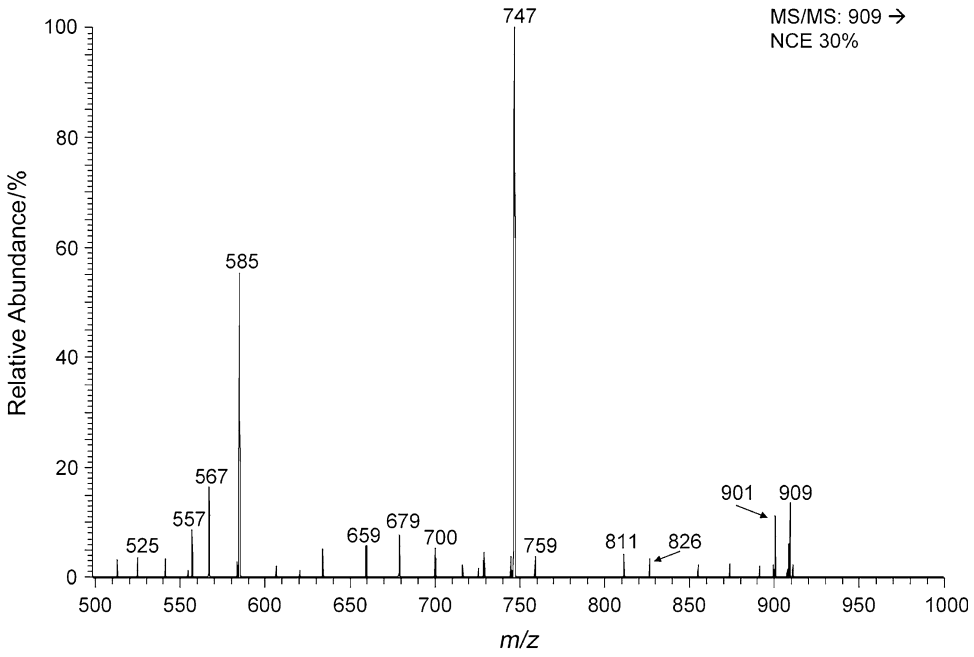

Fig. 6 MS^2 spectrum of a "dimer" product at *m/z* 909

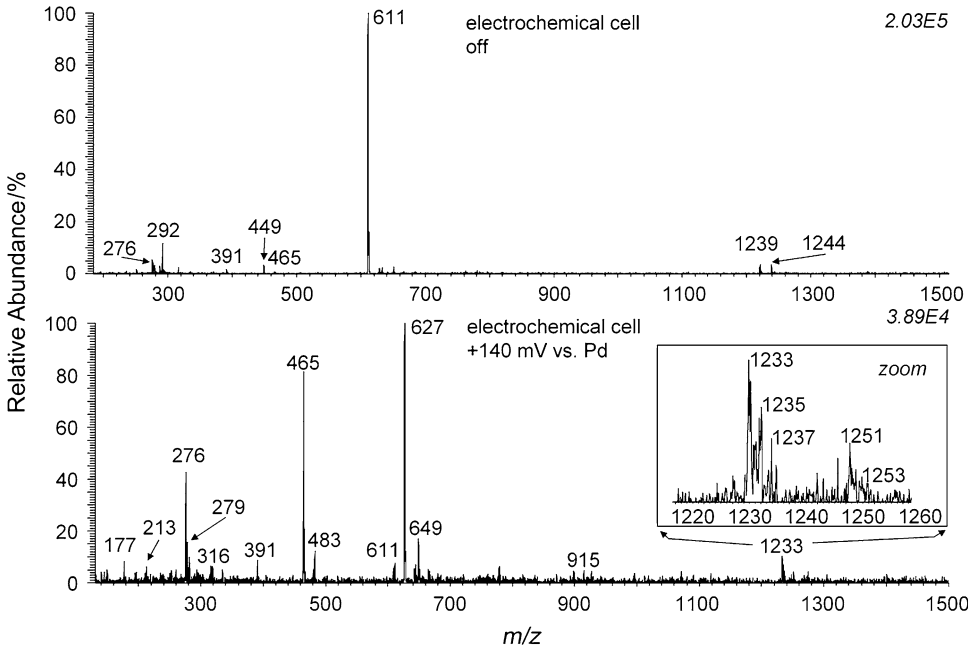

Fig. 7 MS spectra of cyanidin-3,5-diglucoside (*top*) and products of its oxidation (*bottom*; *inset* shows the zoom of product at *m/z* 1,233)

Carl Roth (Karlsruhe, Germany). The concentration of anthocyanin standards used for the experiments was 0.1 mg/cm^3. Trifluoroacetic acid (99%) and acetonitrile (gradient grade) were provided by Fluka (Buchs, Switzerland). The mobile phase (liquid for transportation of the sample zone to the electrochemical cell and ion source of the mass spectrometer) was prepared by mixing deionized water (Elga, Elgastat, Bucks, Great Britain) with acetonitrile and trifluoroacetic acid to give the final mixture (0.12% trifluoroacetic acid, 15% acetonitrile in water, v/v).

Instrumentation

A Finnigan MAT LCQ^{TM} ESI–MS instrument (an ion trap mass spectrometer; Finnigan, San Jose, USA) was used for hyphenation with a Coulochem III electrochemical detector, equipped with a dual-channel coulometric cell (model 5010A) and a guard cell (model 5020) (all ESA Inc., Chelmsford, MA, USA). The liquid used for transport of sample through the electrochemical cell to the ion source was identical with the mobile phase used for measurement

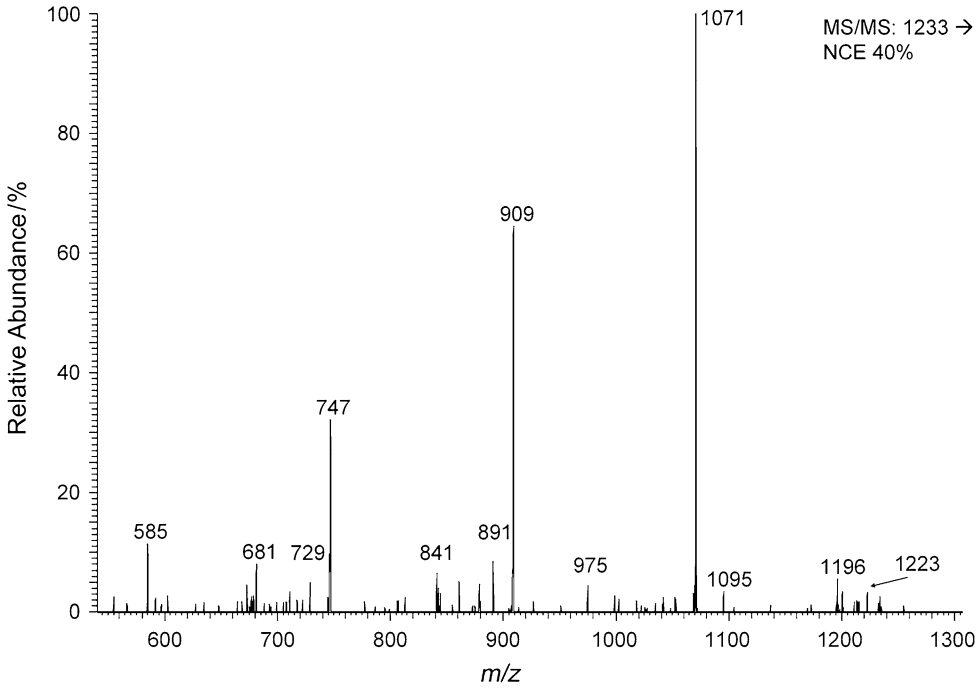

Fig. 8 MS^2 spectrum of oxidized product of cyanidin-3,5-diglucoside at *m/z* 1,233

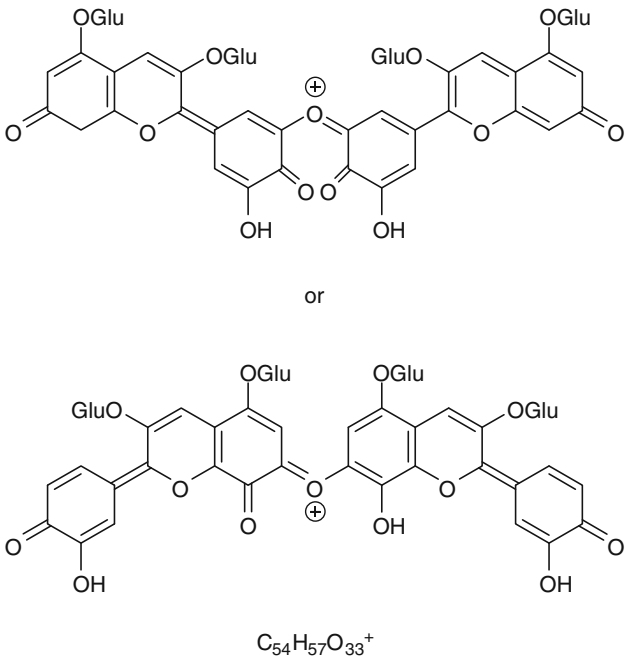

Fig. 9 Speculative structure of products of the oxidative condensation of cyanidin-3,5-diglucoside

of hydrodynamic voltammograms. The sample of anthocyanin was injected into the flow of acidic mobile phase and the zone was pumped into an electrochemical cell. The liquid was pumped through the system using a chromatographic pump Rheos 2000 (Flux Instruments, Reinach, Switzerland). The flow rate used was 0.2 cm^3/min. A volume of 20 mm^3 of sample solution (0.1 mg/cm^3) was injected (full loop). The electrochemical cell was operated at selected voltages within the range 0 to +200 mV (vs. Pd/H_2). The parameters of the ion source and ion optics were tuned using a standard solution of studied pigments. Spray voltage +5.6 kV, sheath gas flow rate 60 arb. (arbitrary units), auxiliary gas 0 arb., and temperature of a heated capillary 200 °C were used. The electrochemical cell was connected to a grounded block before entering the ion source by a PEEK capillary (0.13 mm I.D.). From the grounded block the liquid was introduced to the ion source using a fused silica capillary (0.075 mm I.D.). The mass spectrometer was operated in the full scan mode (range *m/z* 50–1,500). Product ions were identified by online tandem and multi-stage (MS^2 and MS^3) experiments performed by collision-induced dissociation of the studied ion after its isolation in the ion trap.

For hydrodynamic voltammogram measurements, the HPLC system consisted of an ESA isocratic pump (model 582; ESA Inc., Chelmsford, MA, USA) with a pulse damper, a manual injector (Rheodyne, Cotati, CA, USA) equipped with a 10-mm^3 loop and an ESA Coulochem III coulometric detector with a dual-electrode standard analytical cell (model 5010A) combined with a guard cell (model 5020) (all ESA Inc., Chelmsford, MA, USA).

Samples were introduced into the system by a glass 25-mm^3 syringe (Hamilton, Reno, NV, USA). All fittings, ferules, and tubing were made of PEEK™. A Purospher

Star RP-18 (5 μm), 125 × 4-mm-I.D. HPLC column (Merck, Darmstadt, Germany) was used.

Mobile phase composition was acetonitrile/water/trifluoroacetic acid (15:85:0.12, v/v). The mobile phase was vacuum-filtered through a 0.2-μm porous filter (Supelco, Bellefonte, PA, USA) and degassed by helium sparkling prior to use. The flow rate was 1.2 cm^3/min. The working potential was increased gradually in 70-mV increments in the range 100–700 mV (vs. Pd/H_2). Hydrodynamic voltammograms were expressed as a normalized mean peak area vs. applied potential. The chromatographic station Clarity (DataApex, Prague, Czech Republic) was used for chromatogram recording.

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