

Profiling of phenolic glycosidic conjugates in leaves of *Arabidopsis thaliana* using LC/MS

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Received 17 July 2006; accepted 25 July 2006

Profiling of plant secondary metabolites is still a very difficult task. Liquid chromatography (LC) or capillary electrophoresis hyphenated with different kinds of detectors are methods of choice for analysis of polar, thermo labile compounds with high molecular masses. We demonstrate the applicability of LC combined with UV diode array or/and mass spectrometric detectors for the unambiguous identification and quantification of flavonoid conjugates isolated from *Arabidopsis thaliana* leaves of different genotypes and grown in different environmental conditions. During LC/UV/MS/MS analyses we were able to identify tetra-, tri-, and di-glycosides of kaempferol, quercetin and isorhamnetin. Based on our results we can conclude that due to the co-elution of different chemical compounds in reversed phase HPLC systems the application of UV detectors does not allow to precisely profile all flavonoid conjugates existing in *A. thaliana* genotypes. Using MS detection it was possible to unambiguously recognize the glycosylation patterns of the aglycones. However, from the mass spectra we could not conclude neither the anomeric form of the C-1 carbon atoms of sugar moieties in glycosidic bonds between sugars or sugar and aglycone nor the position of the second carbon involved in disaccharides. The applicability of collision induced dissociation techniques (CID MS/MS) for structural analyses of the studied group of plant secondary metabolites with two types of analyzers (triple quadrupole or ion trap) was demonstrated.

KEY WORDS: liquid chromatography-mass spectrometry; metabolite profiling; metabolomics; flavonoid glycosides.

1. Introduction

The synthesis of plant secondary metabolites represents a complex cellular network involving the transcription, translation and post-translational modification of many gene products. Analysis of whole plant metabolomes is a difficult task due to the high number and diversity of primary and secondary metabolites present in plant tissues (Dixon and Strack, 2003; Sumner *et al.*, 2003; Fernie *et al.*, 2004; Stobiecki and Kachlicki 2005). The differences in the physicochemical properties and molecular masses between various classes of compounds (some may exceed 1000 Da) mean that a single technique can generally not separate and identify all of these metabolites simultaneously. Analysis may therefore be carried out by consecutive profiling of different metabolite classes using different chromatographic techniques and detectors. Recent developments in the field of metabolomics now allow the detection of more than 500 metabolites during a single analytical run (Roessner *et al.*, 2001; Sumner *et al.*, 2003).

Flavonoids are widely distributed in the plant kingdom. They constitute an important class of plant secondary metabolites and are mostly present as glycosidic conjugates. Their synthesis is part of the phenylpropanoid pathway and is closely related to the synthesis of phenolic acids, aldehydes and alcohols, which serve as precursors of lignins (Nair *et al.*, 2004; Rogers *et al.*, 2005). Flavonoids play an essential role in various biochemical and physiological processes (Dixon and Paiva, 1995; Taylor and Grotewold, 2005). They contribute to cellular protection against UV light irradiation (Laundry *et al.*, 1995), their radical scavenging properties may help to maintain the cellular redox balance (Pietta, 2000), many of them have phytoalexin or phytoanticipin activities (Van Etten *et al.*, 1994) and they can alter polar auxin transport (Buer and Muday, 2004). Further understanding of the functions of flavonoids in plant physiology and development will require comprehensive studies utilizing functional genomics but it will also be necessary to develop new methods for their accurate detection and quantification. Genetic engineering of these natural products has received a substantial amount of interest as their presence strongly influences the characteristics of plant products that are used for food and feed production (Dixon and Steel, 1999; Verpoorte and Memelink, 2002).

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Flavonoids represent a large group of structurally related compounds with several subclasses such as flavanones, flavones, flavonols, isoflavones, flavan 3-ols and anthocyanidines (Stafford, 1990). Many of these compounds are positional isomers or homologues, which may be distinguished on the basis of different light absorption spectra in the UV-Vis range. Structurally, they differ in the degree of hydroxylation and that hydroxyls may be sometimes methylated. The aglycones are also O- or C-glycosylated, and the degree and the position of glycosylation may vary between plant tissues or species (Harborne and Williams, 2000). Glycosylation of flavonoids leads to an increase of their molecular masses and affects their physicochemical properties and decreases their thermal stability. In this situation liquid chromatography or capillary electrophoresis are methods of choice for the separation of the target compounds (Niessen, 1999). However, hyphenation of these systems with mass spectrometers is much easier to control in the case of LC, therefore LC/MS and/or LC/UV are most often used for profiling flavonoids. Other detectors like electrochemical (Aguilar-Sanchez *et al.*, 2005) or laser induced fluorescence detectors may also be applied (Simeon *et al.*, 2001). Mass spectrometers combined with liquid chromatographs are currently equipped with atmospheric pressure ionization sources (ESI, electro-spray ionization, or APCI, atmospheric pressure chemical ionization). The type of analyzer used in these instruments plays an important role for compound identification. Co-elution of the target compounds frequently occurs during LC profiling of flavonoid conjugates in plant extracts. In this situation even the use of standards for identification will not allow unambiguous results. This problem occurs when UV, UV-DAD or single sector mass analyzer detectors are used. Single sector mass analyzers only register normal mass spectra and additional data from collision induced dissociation are not available. The comparison of the retention times and UV spectra or even molecular masses recorded in mass spectra registered in positive and/or negative ion mode, $[M + H]^+$ or $[M - H]^-$ ions, respectively, does not give sufficient information. In contrast, collision induced dissociation (CID) combined with tandem mass spectrometric techniques (CID MS/MS) allows the successful identification of target compounds. This approach is essential for the structural characterization of compounds present in the mixtures where the mass data obtained is often sufficient for full characterization of the studied compounds. CID MS/MS spectra allow different aglycone glycosylation patterns and isomeric aglycones (flavones, flavonols and isoflavones) to be distinguished. Mass spectrometric analysis of purified flavonoid conjugates as well as those present in mixtures is a well established method (Stobiecki 2000; de Rijke *et al.*, 2003; Cuyckens and Claeys, 2004; Prasain *et al.*, 2004; Stobiecki and Kachlicki, 2006).

It has been reported that in *Arabidopsis thaliana* there are about 15 flavonoid compounds (D'Auria and Gershenzon, 2005). However, after isolation and purification of flavonoids from green tissues of *A. thaliana* many fewer compounds have been fully structurally characterized with various physicochemical methods. Kaempferol 3-O- β -[β -D-glucosyl(1-6)D-glucoside]-7-O- α -L-rhamnoside, kaempferol 3-O- β -D-glucoside-7-O- α -L-rhamnoside, kaempferol 3-O- α -L-rhamnoside]-7-O- α -L-rhamnoside were identified in the Wassilewskija ecotype (Veit and Pauli, 1999). It seems that kaempferol 3-O- β -[α -L-rhamnosyl (1-2) D-glucoside]-7-O- α -L-rhamnoside was also detected in the Columbia ecotype (Bloor and Abrahams, 2002). In addition to flavonol conjugates, the presence of flavan-3-ol monomer and polymers was reported in seeds of *A. thaliana* after careful LC/MS analyses (Routaboul *et al.*, 2006). The second class of flavonoids identified in *A. thaliana* are anthocyanins, with one derivative of cyanidin of molecular mass 1343 Da being characterized (Bloor and Abrahams, 2002).

Flavonoid glycoconjugates isolated from various wild type and transgenic plants of *A. thaliana* plants and seeds were analyzed with LC systems equipped with different detectors. Several reports describe the profiling of flavonol glycosides in different wild type and/or genetically transformed *A. thaliana* ecotypes (Columbia and Wassilewskija). An LC/UV system was used to profile flavonoid glycosides and products of their hydrolysis in *A. thaliana* seedlings to investigate the role of individual proteins in flavonoid biosynthesis. In this study only aglycones (quercetin and kaempferol) present in the acidic hydrolysates of extracts were identified after comparison of retention times observed for aglycone standards (Pelletier *et al.*, 1999). Six di- and triglycosides of kaempferol and quercetin were proposed for the wild type Columbia seedlings (Graham, 1998). Di- and triglycosides of both mentioned flavonols were identified in green parts and flowers of *A. thaliana* plants with altered expression of flavonol glycosyltransferase genes (Jones *et al.*, 2003). Two quercetin and four kaempferol glycosides as well as seven anthocyanins were identified in transgenic tomato fruits that had been modified to simultaneously express two heterologous maize transcription factors known to regulate flavonoid accumulation (Le Gall *et al.*, 2003). Authors of another paper reported the identification of six flavonol glycosides and eleven anthocyanins in extracts of green parts of *A. thaliana* seedlings, over-expressing a MYB transcription factor (Tohge *et al.*, 2005). During these studies two different MS systems were used for targeted and non-targeted secondary metabolite identification. Another research group used HPLC coupled to UV spectral analysis to study flavonol glycosides in *A. thaliana* plants over-expressing or under-expressing the R2R3-MYB transcription factor MYB12 (Mehrtrens *et al.*, 2005). In some of the above-mentioned papers

authors reported the presence of sinapate esters which were recognized in the registered chromatograms (Le Gall *et al.*, 2005; Tohge *et al.*, 2005). Profiles of secondary metabolites were also studied during the interaction of *A. thaliana* with pathogenic microorganism. In UV chromatograms authors were able to detect four kaempferol glycosides and sinapoyl malate, and their presence was confirmed by LC/MS analyses (Kliebenstein *et al.*, 2005). Structures of phenolic compounds identified in green parts of Arabidopsis plants are presented on Scheme 1. The application of LC-MS also allowed the flavonoid conjugates composition to be established (flavonols, flavan 3-ol and procyanidins) in seed coats of wild type (ecotypes: Wassilewskija-2, Columbia-0 and Landsberg *erecta*) and mutant *A. thaliana* plants (Pourcel *et al.*, 2005; Routaboul *et al.*, 2006). The method of unambiguous identification of these compounds will be published elsewhere (Kerohas *et al.*, accepted). The confidence of the identification strongly depends on the type of detector used during the LC, which is particularly important in the case of profiling of flavonoid conjugates isolated from different lines of wild type and transgenic *A. thaliana* plants. *A. thaliana* is the model species for plant molecular genetics but its utility for studying the physiological role of flavonoids is limited as there is an urgent need for unified analytical procedures for the reproducible and accurate identification of flavonoid conjugate profiles. Here we demonstrate the applicability of LC combined with UV and mass spectrometric detectors for profiling flavonoid conjugates in extracts obtained from green parts of *A. thaliana* plants (ecotype C24). The studied extracts were obtained from the wild type and a line of genetically modified plants. We compared results obtained with two different mass spectrometers operat-

ing with electro-spray ionization. The first system was equipped with a triple quadrupole and the second with an ion trap analyzer. The strategy of the registration and interpretation of CID MS/MS spectra obtained with both MS systems is presented. The use of tandem mass spectrometric techniques improves in some extent structural characterization of flavonoid metabolites.

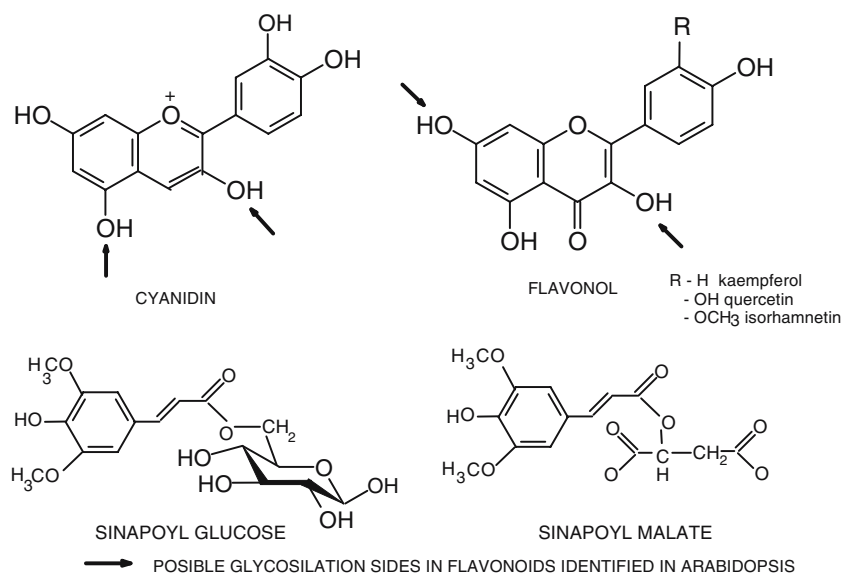
2. Materials and methods

2.1. Plant material

A. thaliana (L.) Heynh. seeds of ecotype C24 were sown in soil (Einheitserde GS90; Gebrüder Patzer, Sinntal-Jossa, Germany) and grown in a growth chamber with a 16-h day length provided by fluorescent light at 80 or 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a day/night temperature of 20/16 °C and relative humidity of 60/75%. In addition to the wild type (samples AR1 and AR24/25) transgenic plants with reduced expression of a plant transcription factor gene (sample AR49/50; Skiryicz *et al.*, unpublished data) were used. Samples AR24/25 and AR49/50 were obtained from plants kept in a 4 °C growth chamber with a 16-h photoperiod at 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 5 days before the sample collection. The transgenic line and the cold treatment were included in our experimental setup to test whether changes in phenolic glycosidic conjugates would be detectable with our analytic system. Whole rosettes pooled from two to three plants were harvested after approximately 4 h of light.

2.2. Isolation of phenolic compounds

Plant material was frozen in liquid nitrogen and powdered in a Retsch mill. For the extraction of



Scheme 1. Structures of flavonoid conjugates and sinapic acid esters identified in *A. thaliana*.

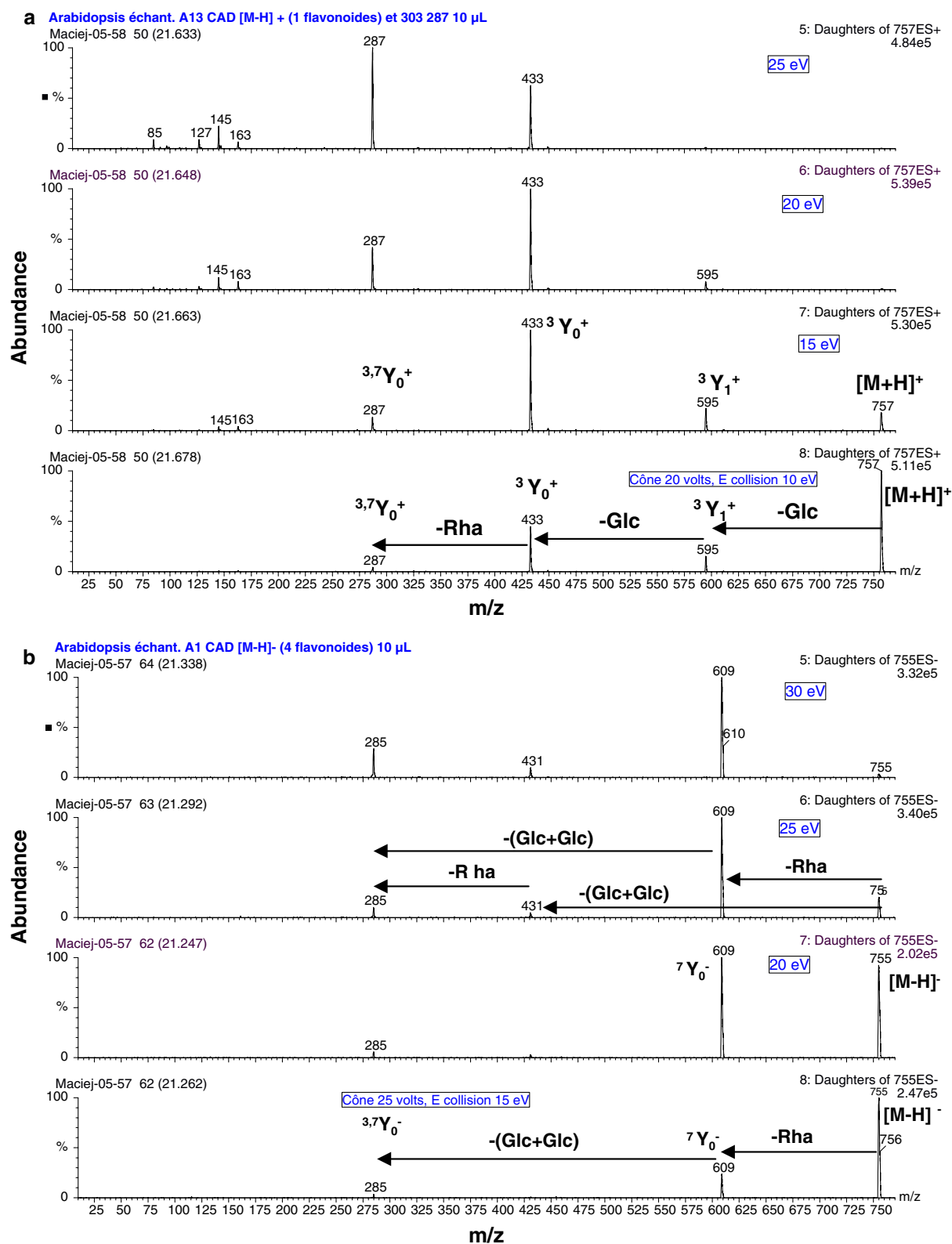


Figure 1. CID mass spectra of kaempferol 3-*O*-glucosyl-glucoside 7-*O*-rhamnoside, MW 756: (a) Product ion from [M + H]⁺ ions – cone voltage 20 V and collision energy: 10, 15, 20 and 25 eV, registered with triple quadrupole analyzer, (b) product ion from [M – H]⁻ ions – cone voltage 25 V and collision energy: 15, 20, 25 and 30 eV, registered with triple quadrupole analyzer, (c) CID MSⁿ product ion spectra registered with ion trap analyzer in positive ion mode, (d) CID MSⁿ product ion spectra registered with ion trap analyzer in negative ion mode.

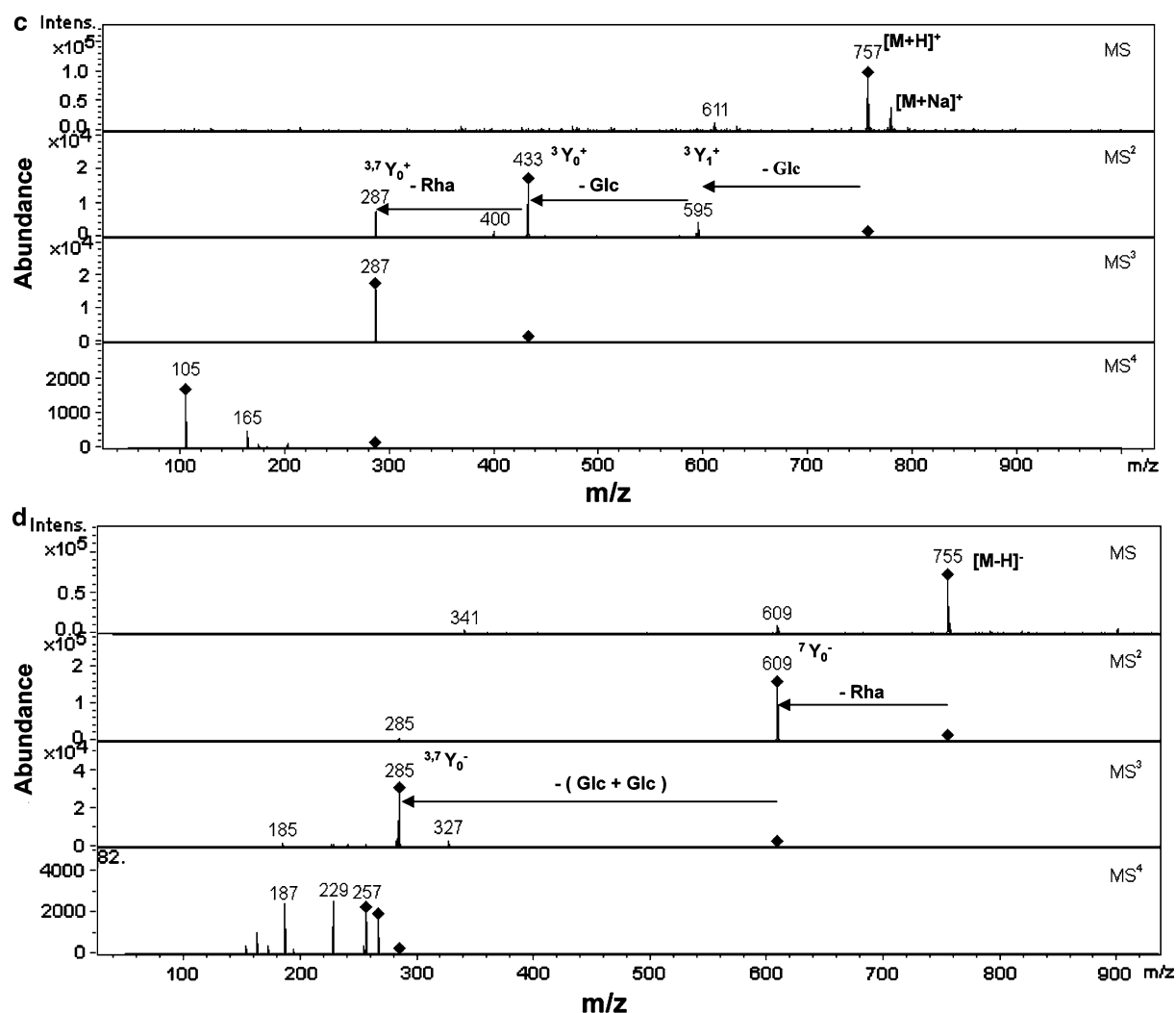


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flavonoid glycosides plant material (~ 1 g fresh weight per sample AR1N, AR24/25N and AR49/50N) was homogenized in 10 mL of 80% (v/v) methanol, and the suspensions were placed in an ultrasonic bath for 30 min. Genistein was added as an internal standard to each analyzed sample prior to extraction procedure, the final concentration of the standard in the samples injected into the LC column being about $0.05 \mu\text{g}/\mu\text{L}$. The extracts were filtered through a Büchner funnel and concentrated under reduced pressure on a rotovapour at a bath temperature of 40°C . The dried sample was dissolved in $500 \mu\text{L}$ of 50% methanol and transferred to the LC vial, the flask was washed with the next portion of $500 \mu\text{L}$ of 50% methanol and both volumes were combined in the vial. The efficiency of extraction of anthocyanins from leaf tissue was increased by using 80% MeOH acidified to $\text{pH} = 3$ with acetic acid (sample AR1H⁺). In this procedure extracts were neutralized with 2 N ammonia after the sonification, centrifugation and filtration. Further treatment (preparation) of the neutralized extract was as described above.

2.3. Liquid chromatography

LC analyses of plant extracts were performed with two different LC systems combined with two different types of mass spectrometers (see below). One instrument used was an Agilent 1100 HPLC pump (Palo Alto, CA, USA), where the samples were injected through a $5 \mu\text{L}$ injection loop onto a Superspher 100 RP-18 column (250×2 mm; Merck), at a 0.2 mL/min flow rate. The second system was equipped with an HPLC pump, UV detector and automatic injector (model Alliance, Waters, Bedford, MA, USA); the injection volume was $10 \mu\text{L}$, the same LC column and flow were used during the analyses.

During LC, the elution from the LC column was carried out using mixtures of two solvents: A (95% acetonitrile, 4.5% H₂O, 0.5% formic acid, v/v/v) and B (95% H₂O, 4.5% acetonitrile, 0.5% formic acid v/v/v). The elution steps were as follows: 0–5 min isocratic at 10% A, 5–40 min linear gradient from 10 to 30% of A, 40–48 min linear gradient up to 100% of A, 48–60 min isocratic at 100% of A. Isoflavonoids were identified by

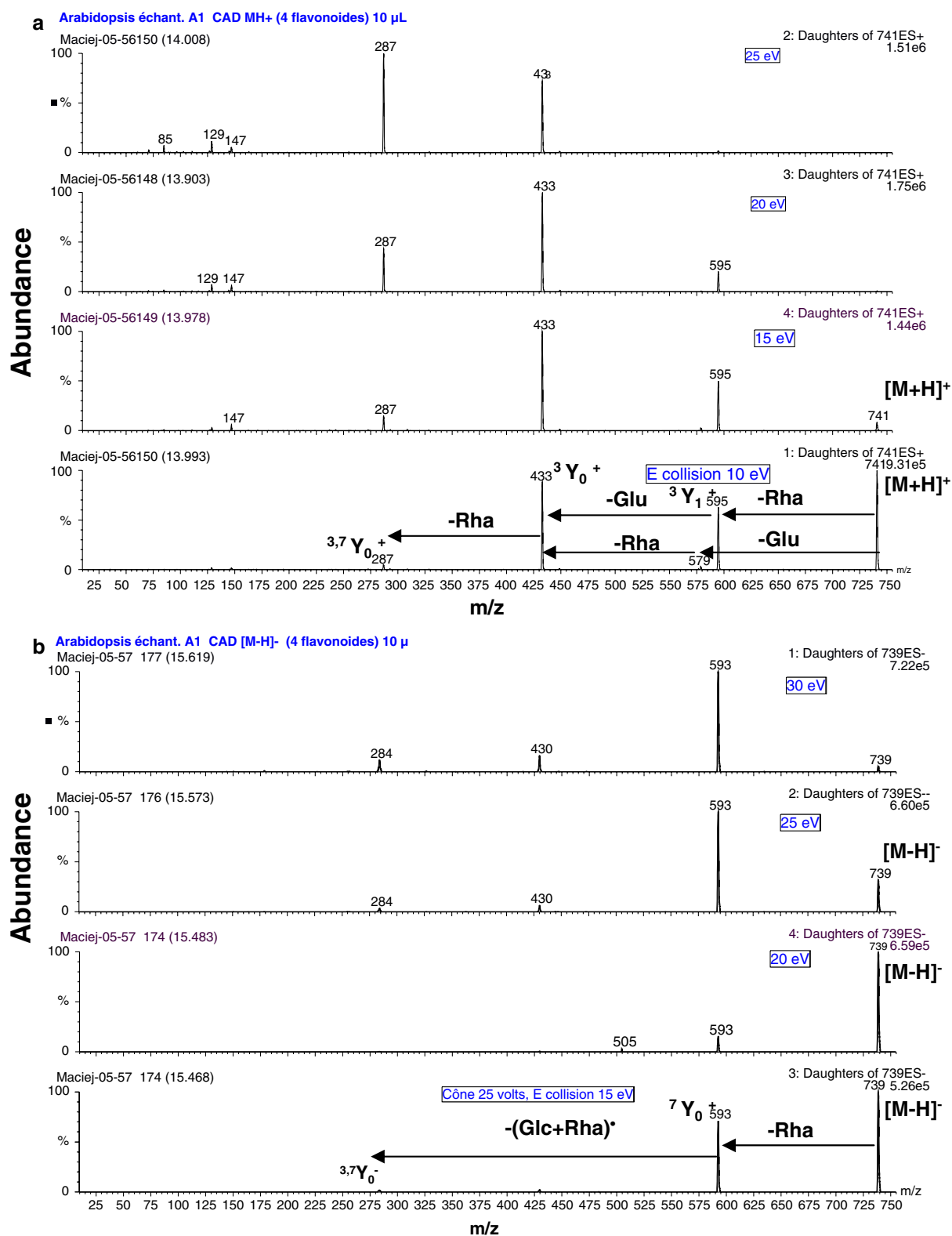


Figure 2. CID mass spectra of kaempferol 3-*O*-rhamnosyl glucoside 7-*O*-rhamnoside, MW 740: a. [M+H]⁺ ions – cone voltage 20 V and collision energy: 15 eV, 20 eV and 25 eV, registered with triple quadrupole analyzer, b. [M-H]⁻ ions – cone voltage 25 V and collision energy: 15 eV, 20 eV, 25 eV and 30 eV, registered with triple quadrupole analyzer, c. CID MSⁿ product ion spectra registered with ion trap analyzer in positive ion mode, d. CID MSⁿ product ion spectra registered with ion trap analyzer in negative ion mode.

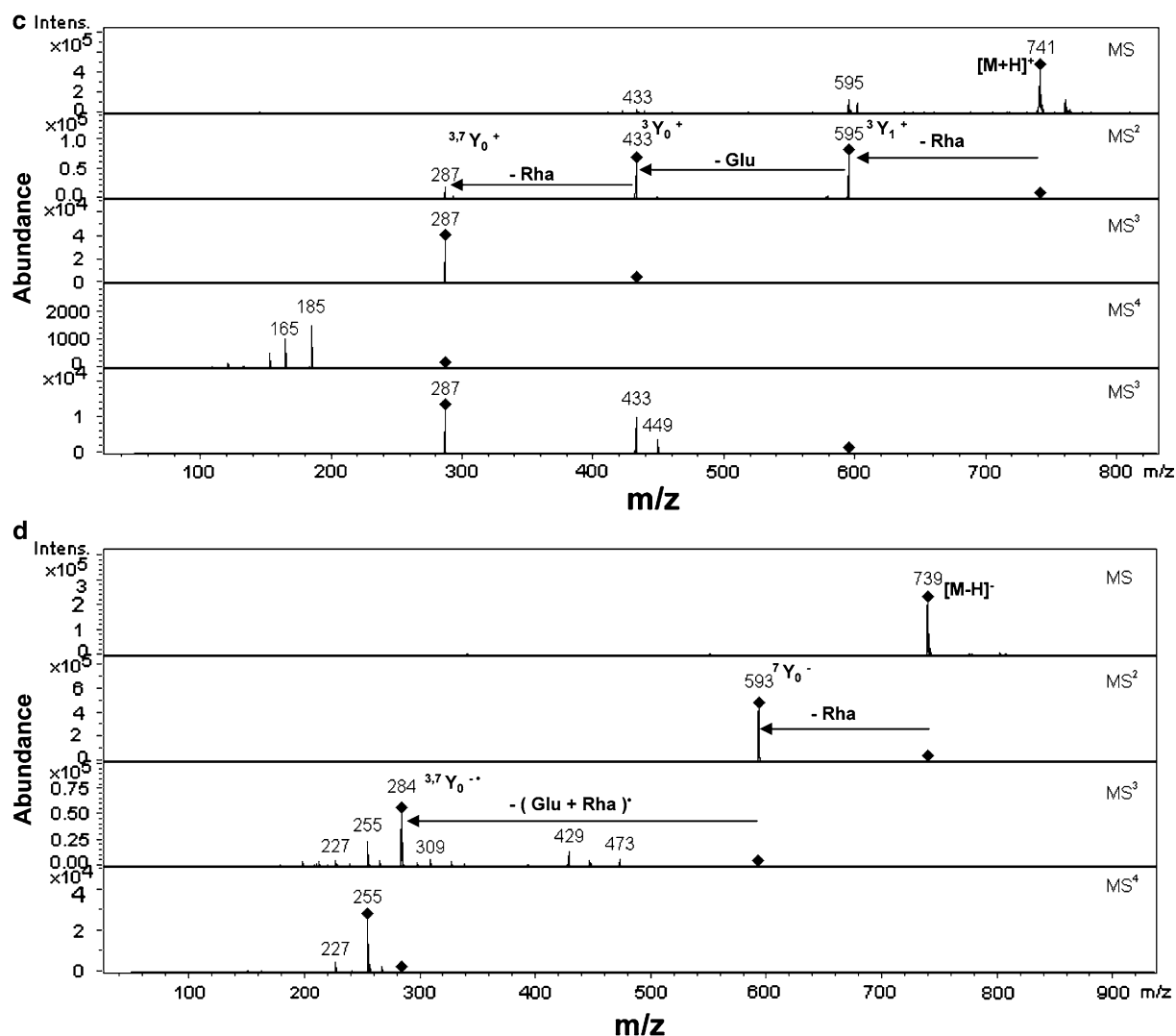


Figure 2. Continued.

their MS spectra and/or by their retention times compared with the data obtained for standards. To improve the separation of isoflavone diglucosides, the linear gradient was in some experiments started immediately from 0% A to 30% A in 30 min, then increased to 100% A between 30 and 35 min and 100% of A was maintained from 35 to 50 min.

2.4. Liquid chromatography–mass spectrometry (LC/ESI/MSⁿ) analyses

LC/ESI/MSⁿ analyses were performed in two different labs, on an ion trap (IT) mass spectrometer, model Esquire 3000 (Bruker Daltonics, Bremen, Germany) in Poznań, and a triple quadrupole mass spectrometer Quattro LCTM (Micromass, Manchester, UK) at INRA Versailles, France. Both mass spectrometers were equipped with electrospray ionization (ESI) sources. IT source parameters were as follows: ESI source voltage of 4 kV, nebulization with nitrogen at

30 psi, dry gas flow of 9.0 l/min at temperature of 310 °C, skimmer 1 voltage 12.4 or 44.2 V, collision energy set to 30%. The instrument operated under EsquireControl version 5.1, and data were analyzed using the Data Analysis version 3.1 package delivered by Bruker. The triple quadrupole ESI source potentials were as follows: capillary 3.25 kV (positive mode) or 3.0 kV (negative mode) and extractor 2 V. The sampling cone voltage was varied usually from 20 to 50 V for ESI mass spectra. The specific value of the cone voltage and ion energies during CID experiments were set in order to optimize the intensity of the parent ion. The quadrupole mass filters were set with LM and HM resolution of 15.0 (arbitrary units), which is equivalent to a 1.0 Da-mass window for transmission of both precursor and product ions. The source block and desolvation temperatures were set at 120 and 400 °C, respectively. Argon was used as collision gas at 3.5×10^{-3} torr. Data acquisition and processing were carried out using software MassLynx version 4.0.

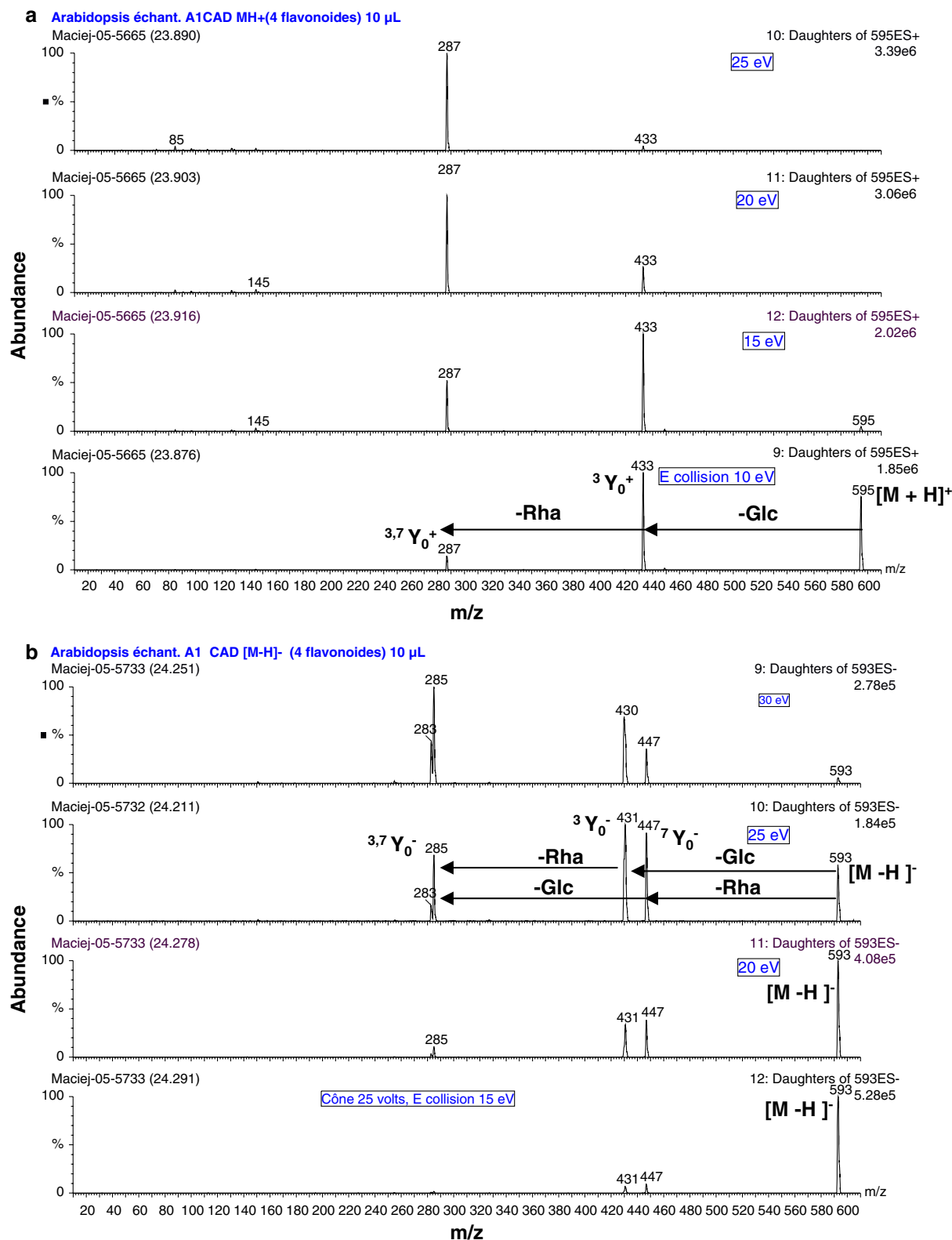


Figure 3. CID mass spectra of kaempferol 3-*O*-glucoside 7-*O*-rhamnoside, MW 594: (a) $[M + H]^+$ ions – cone voltage 20 V and collision energy from 10 to 25 eV, registered with triple quadrupole analyzer, (b) $[M - H]^-$ ions – cone voltage 25 V and collision energy from 15 to 30 eV, registered with triple quadrupole analyzer, (c) CID MSⁿ product ion spectra registered with ion trap analyzer in positive ion mode, (d) CID MSⁿ product ion spectra registered with ion trap analyzer in negative ion mode.

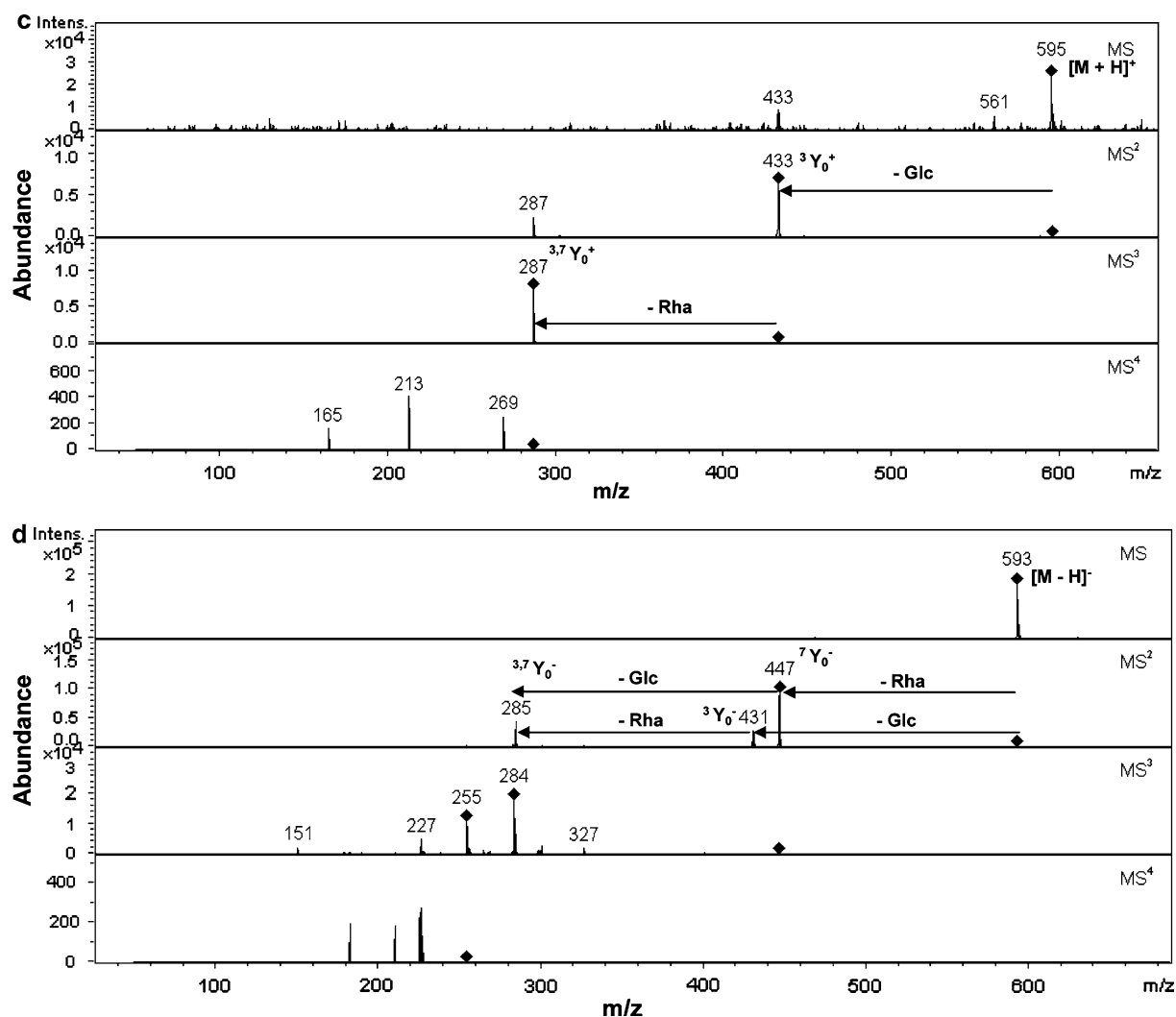


Figure 3. Continued.

3. Results and discussion

During our studies on phenolic secondary metabolites (especially flavonoid conjugates) in *A. thaliana* we analyzed extracts obtained from leaves. Wild type plants (ecotype C24) as well as transgenic line exhibiting changed expression level of a so-far uncharacterized transcription factor (Skirycz *et al.*, unpublished data) were grown at normal temperature or subjected to cold treatment for 5 days (for details see Materials and methods). Flavonoid conjugates were extracted from leaves using 80% methanol. In comparative studies, leaf material of wild type plants grown under normal conditions was extracted with 80% methanol acidified with acetic acid to pH = 3. The quality of the information on the structures of the compounds analyzed depended on the detector type applied in the liquid chromatograph. One system consisted of an LC pump and UV diode array detector recording UV spectra in the range from 200 to 400 nm. The two other systems were LC/MS instruments located in two different labs (both with

electrospray ionization): one mass spectrometer was equipped with a triple quadrupole analyzer (lab at INRA Versailles, France) and the second with an ion trap (lab at Institute of Plant Genetics PAS, Poznań, Poland).

3.1. Choice of ionization conditions for sample analysis

Atmospheric pressure ionization techniques (ESI – electrospray ionization, or APCI – atmospheric pressure chemical ionization) are at present the methods of choice applied in LC/MS systems used for metabolite profiling. Ions created during the ionization processes (protonated $[M + H]^+$ or deprotonated $[M - H]^-$ molecule ions) have low internal energy, so their fragmentation in the ion source is not intense and strongly depends on potentials applied in the ion source. Low values of the potential between capillary and entrance slit of the analyzer allow to register mass spectra containing signals mainly corresponding to these $[M + H]^+$

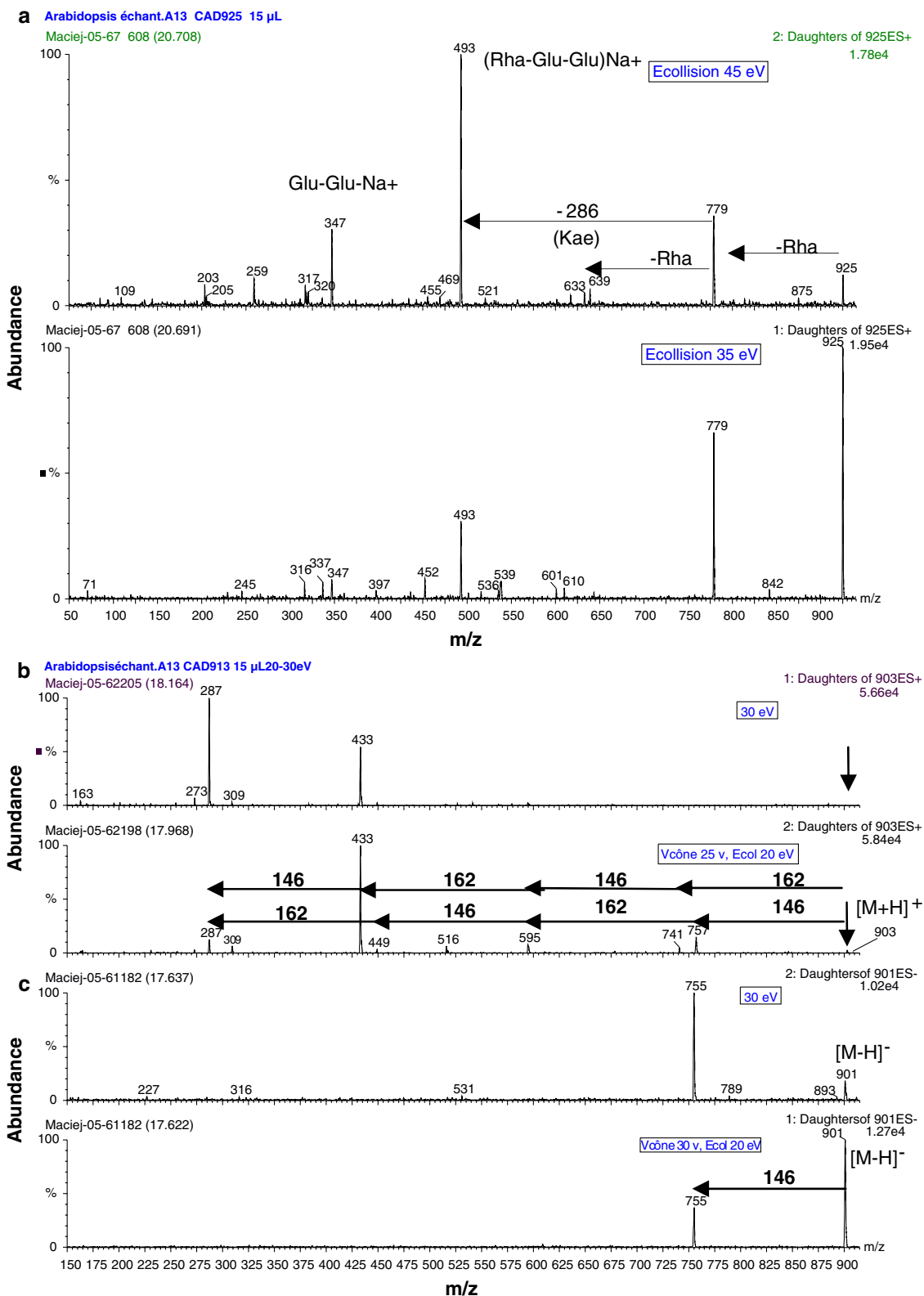


Figure 4. CID MS/MS spectra of kaempferol 3-*O*-rhamnosyl-glucosyl-glucoside 7-*O*-rhamnoside, MW 902: (a) $[M + Na]^+$ ions – cone voltage 25 V and collision energy 20 or 25 eV, registered with triple quadrupole analyzer, (b) $[M + H]^+$ ions – cone voltage 25 V and collision energy 20 or 30 eV, registered with triple quadrupole analyzer, (c) $[M - H]^-$ ions – cone voltage 30 V and collision energy 20 or 30 eV, registered with triple quadrupole analyzer, (d) CID MSⁿ product ion spectra of $[M + H]^+$ ion, registered with ion trap analyzer, (e) CID MSⁿ product ion spectra of $[M - H]^-$ ion, registered with ion trap analyzer.

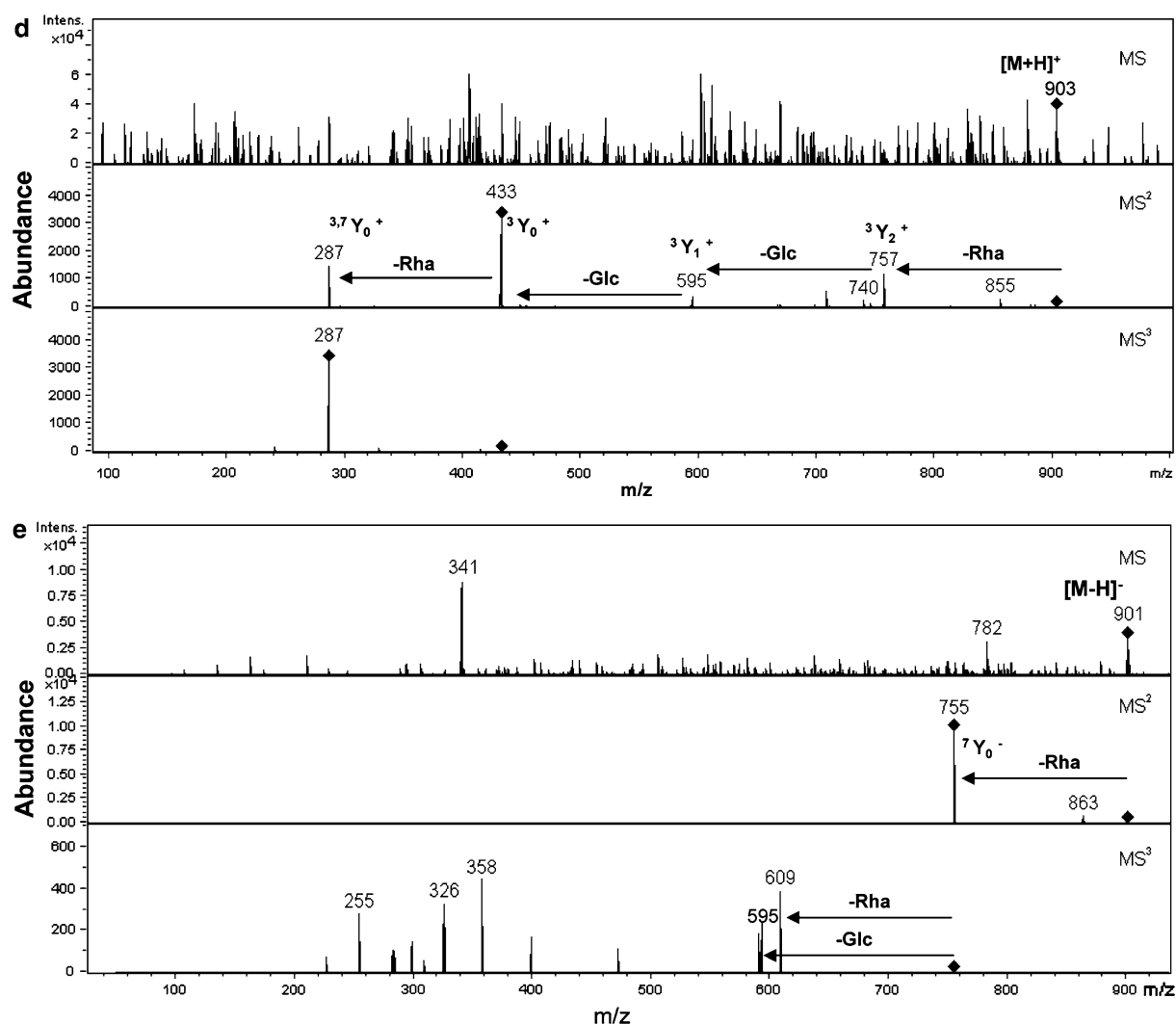
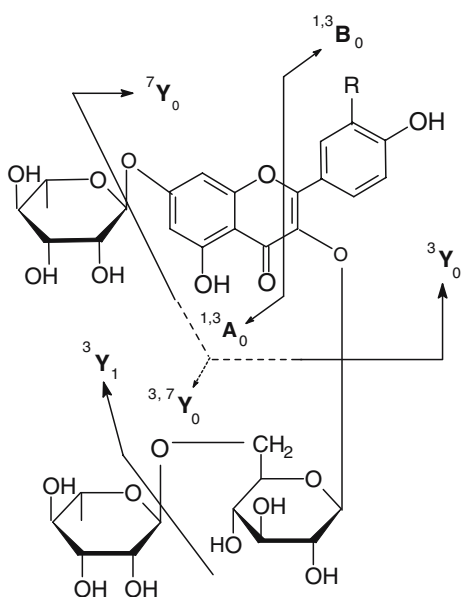


Figure 4. Continued.

or $[M-H]^-$ ions (see figures 1–4). Additionally, co-elution of different secondary metabolites from the LC column is a common phenomenon occurring during the analyses of raw extracts from plant tissues. This results in first order mass spectra showing numerous ions representing deprotonated or protonated molecules created after ionization of different substances. In this situation, for a proper analysis of complex mixtures of secondary metabolites present in plant tissues it is necessary to apply mass spectrometric techniques allowing the registration of fragment ions characteristic for the analyzed compounds. In most cases, this goal is achieved by the application of CID in combination with different tandem mass spectrometric techniques (CID MS/MS) (Jennings, 1996; March *et al.*, 2004). The registration of CID spectra in the positive and/or negative ion mode permitted the structural characterization of the analyzed flavonoid conjugates (Justensen *et al.*, 1998; Waridel *et al.*, 2001; de Rijke *et al.*, 2003, 2004; Kachlicki *et al.*, 2005; Le Gall *et al.*, 2005; Zhang and Brodbelt, 2005; Zhang *et al.*, 2005).

To optimize the performance of mass spectrometers during CID MS/MS analyzes it is necessary to control several parameters of ionization and ion optics. In the case of the triple quadrupole analyzer different cone voltages (potential between the tip of the capillary and the entrance slit to the analyzer) were applied for performing CID experiments on $[M+H]^+$ ions and aglycone fragment ions ($^{3,7}Y_0^+$), see Scheme 2. The application of higher cone voltage caused higher intensity aglycone ions in the first order mass spectra, due to the fragmentation occurring in the ion source. The ions were further collided with argon atoms in the second quadrupole and the product ions spectra resolved in the third quadrupole had a good signal to noise ratio. The optimization of collision energy is also necessary, allowing the degree of fragmentation and intensities of consecutive registered product ions to be controlled. In the case of the ion trap spectrometer the skimmer voltage plays a role similar to that of the cone voltage of triple quadrupole instruments and its increase resulted in the decrease of $[M+H]^+$ or $[M-H]^-$ flavonoid



Scheme 2. Description of product ions observed in the mass spectra registered in positive and negative ion mode after cleavages of glycosidic bonds between sugars or sugar and the aglycone. The nomenclature of the fragment ions was proposed by Domon and Costello (1988).

glycoside ion intensities. Ions of protonated or deprotonated molecules are subjected to collisions with helium atoms during the registration of mass spectra in the CID mode. The energy applied to ions during the CID process may also be optimized, however, in the case of the ion trap changes of this parameter have a lower influence on the yield of product ions than in triple quadrupole instruments. In consecutive MS^n experiments the most intense product ions are chosen for further collisions. During our studies it was possible to repeat collision experiments with product ions of proper intensity even in five consecutive steps. To draw conclusions about the structure of the phenolic compounds in *A. thaliana* extracts it was necessary to run CID MS spectra on $[M+H]^+$, $[M+Na]^+$ or $[M-H]^-$ as well as the aglycone fragment ions: $[^{3,7}Y_0^+]$ or $[^{3,7}Y_0^-]$. The general fragmentation pattern of flavonol glycosides after ESI ionization is presented on Scheme 2 and the nomenclature applied for description of fragmentation pathways was proposed by Domon and Costello (1988). The information about structures of flavonoid conjugates identified earlier in *A. thaliana* (Veit and Pauli, 1999; Bloor and Abrahams, 2002; Toghe et al., 2005; Routaboul et al., 2006; Kerhoas et al., accepted) was very helpful for our studies.

3.2. Identification of flavonoid glycoconjugates in Arabidopsis leaves

The application of LC/UV and both above mentioned LC/ MS^n systems permitted the identification of nine flavonol glycosides in extracts obtained from the

wild type (AR1, AR24/25) and transformed line (AR49/50) of *A. thaliana* plants. These compounds were tetra-, tri- and diglycosides of kaempferol, quercetin and isorhamnetin. Additionally, the presence of several glycosides (tri- and tetra-) of cyanidine acylated with different aromatic and/or aliphatic acids was confirmed (table 1). In addition to the flavonoid conjugates also sinapoyl esters were recognized in the registered chromatograms. The detection and identification of these compounds is detailed below.

3.2.1. LC/UV detection

In the *A. thaliana* plants cultivated at normal or decreased temperatures kaempferol glycosides were the main phenolic secondary metabolites in the leaf extracts. Peaks originating from flavonoid glycosides and sinapoyl esters were observed at $\lambda = 350$ nm in the UV chromatograms (figure 5). Glycosides of quercetin or isorhamnetin and anthocyanins co-eluted with kaempferol derivatives and peaks corresponding to some of these compounds were not resolved on the UV chromatograms. Additionally, peaks originating from minor compounds had very low intensities. From the UV spectra registered by the DAD detector at 200–400 nm it was not possible to differentiate between flavonol glycosides and anthocyanins co-eluting under the same peaks of the chromatograms as the detector of the HPLC instrument only measured absorption in the range of UV light. The characteristic absorption wavelengths for anthocyanins are in the visible light (absorbance maxima between 520 and 540 nm).

3.2.2. LC/MS detection

It was possible to estimate the relative amounts of target metabolites in the analyzed leaf extracts on the basis of the abundances of the consecutive peaks in the single ion chromatograms corresponding to $[M+H]^+$ or $[M-H]^-$ ions registered after LC/MS analyses (figure 6). In agreement with the UV detection, the concentrations of kaempferol conjugates in both normal and acidified extracts obtained from wild type C24 ecotype plants were more than one order of magnitude higher than those observed for quercetin or isorhamnetin glycosides as well as for anthocyanins. This ratio was changed in favor of quercetin and cyanidin glycosides in wild type and transgenic *A. thaliana* plants grown in low temperature (table 1). The LC/MS analyses did not allow to unambiguously confirm the structures of the target compounds from the first order mass spectra in which intense $[M+H]^+$ or $[M-H]^-$ and $Y_n^{+/-}$ ions were recorded. Fragment ions were difficult to recognize in the first order mass spectra of minor compounds, especially at low values of potential between the entrance slits to the analyzer in both types of mass spectrometers applied during our studies (data not shown).

Table 1
Flavonoid conjugates identified in extracts of different lines of *Arabidopsis* plants

No	Compound identified	MW	Rt [min]	Relative amount				Reference and ecotype
				AR1N	AR1H ⁺	AR24/25N	AR49/50N	
F1	Kaempferol 3- <i>O</i> -rhamnonyl-glucosyl-glucoside 7- <i>O</i> -rhamnoside	902	21.9	2×10 ⁴	1×10 ⁵	6×10 ³	2×10 ³	New compound
S1	Sinapoyl glucose	386	22.2	3×10 ⁴	2×10 ⁵	1×10 ⁴	2×10 ³	Le Gall et al. (2005) Ws-0; Tohge et al. (2005) Col-0
F2	Quercetin 3- <i>O</i> -rhamnonyl-glucoside 7- <i>O</i> -rhamnoside	756	23.3	1×10 ⁴	traces	4×10 ⁴	4×10 ⁴	Graham (1998) Col-0; Le Gall et al. (2005) Ws-0; Tohge et al. (2005) Col-0
F3	Kaempferol 3- <i>O</i> -rhamnonyl-glucoside 7- <i>O</i> -rhamnoside	740	24.1	1×10 ⁶	8×10 ⁵	2×10 ⁵	2×10 ⁵	Bloor et al. (2002) Col-7; Graham (1998) Col-0; Jones (2003) Col-0;
F4	Quercetin 3- <i>O</i> -glucosyl-glucoside 7- <i>O</i> -rhamnoside	772	24.6	ND	3×10 ⁴	3×10 ⁴	2×10 ⁴	Tohge et al. (2005) Col-0; Veit et al. (1999) Ws-2
F5	Quercetin 3- <i>O</i> -glucoside 7- <i>O</i> -rhamnoside	610	26.3	2×10 ⁴	1.5×10 ⁴	3×10 ⁵	3×10 ⁵	Graham (1998) Col-0
A3	Cyanidin 3- <i>O</i> -[2''- <i>O</i> -(xylosyl) 6''- <i>O</i> -[<i>p</i> - <i>O</i> -(glucosyl)-coumaroyl]-glucoside] 5- <i>O</i> -glucoside	1051	26.3	ND	5×10 ³	ND	6×10 ³	Graham (1998) Col-0; Le Gall et al. (2005) Ws-0; Tohge et al. (2005) Col-0
F6	Kaempferol 3- <i>O</i> -glucosyl-glucoside 7- <i>O</i> -rhamnoside	756	27.2	1.5×10 ⁶	2×10 ⁶	1.5×10 ⁵	4.5×10 ⁴	Klibenstein et al. Col-0
F7	Kaempferol 3- <i>O</i> -glucoside 7- <i>O</i> -rhamnoside	594	28.7	1.5×10 ⁶	2.5×10 ⁶	1×10 ⁶	1×10 ⁶	Bloor et al. (2002) Col-7; Graham (1998) Col-0; Jones (2003) Col-0; Klibenstein et al. (2003) Col-0;
F8	Isorhamnetin 3- <i>O</i> -glucoside 7- <i>O</i> -rhamnoside	624	28.8	1×10 ⁴	ND	2×10 ⁴	1×10 ⁴	Le Gall et al. (2005) Ws-0; Pelletier et al. (1999) Let;
A1	Cyanidin 3- <i>O</i> -[2''- <i>O</i> -[6''- <i>O</i> -(sinapoyl) xylosyl] 6''- <i>O</i> -[<i>p</i> - <i>O</i> -glucosyl]- <i>p</i> -coumaroyl] glucoside] 5- <i>O</i> -malonyl-glucoside	1343	29.9	ND	3×10 ⁴	5×10 ³	4×10 ⁴	Tohge et al. (2005) Col-0; Veit et al. (1999) Ws-2
F9	Kaempferol 3- <i>O</i> -rhamnoside 7- <i>O</i> -rhamnoside	578	31.4	1×10 ⁶	1×10 ⁶	2×10 ⁵	2×10 ⁵	Routaboul et al. (2006) Ws-2
A5	Cyanidin 3- <i>O</i> -[2''- <i>O</i> -(xylosyl) 6''- <i>O</i> -(<i>p</i> -coumaroyl) glucoside]-5- <i>O</i> -glucoside	889	32.9	ND	3.5×10 ⁴	3.5×10 ⁴	4×10 ⁴	Bloor et al. (2002) Col-7; Graham (1998) Col-0; Jones et al. (2003) Col-0; Klibenstein et al. Col-0;
S2	Sinapoyl malate	340	33.2	4×10 ⁴	1.5×10 ⁴	5×10 ³	4×10 ⁴	Le Gall et al. (2005) Ws-0; Pelletier et al. (1999) Let;
A4	Cyanidin 3- <i>O</i> -[2''- <i>O</i> -(xylosyl) 6''- <i>O</i> -(<i>p</i> -coumaroyl) glucoside]-5- <i>O</i> -malonyl-glucoside	975	34.7	ND	1.5×10 ⁴	1×10 ⁴	2×10 ³	Routaboul et al. (2006) Ws-2; Tohge et al. (2005) Col-0; Veit et al. (1999) Ws-2;
A2	Cyanidin 3- <i>O</i> -[2''- <i>O</i> -(6''- <i>O</i> -(sinapoyl) xylosyl) 6''- <i>O</i> -(<i>p</i> -coumaroyl) glucoside] 5- <i>O</i> -malonyl-glucoside	1181	36.8	ND	2×10 ⁴	1×10 ⁴	8×10 ³	von Roepenack-Lahaye et al. (2004) Col-0

AR1N: *A. thaliana*, ecotype C24 wild type, grown at normal temperature, neutral extract (AR1).

AR1H⁺: *A. thaliana*, ecotype C24 wild type, grown at normal temperature, acidic extract (AR1).

AR24/25N: 4 °C: *A. thaliana*, ecotype C24 wild type, grown in 4 °C for 5 days, neutral extract (AR24/25).

AR49/50N: 4 °C, Sup TF: *A. thaliana*, ecotype C24 with suppressed transcription factor expression, grown in 4 °C for 5 days, neutral extract (AR49/50).

ND: not detected.

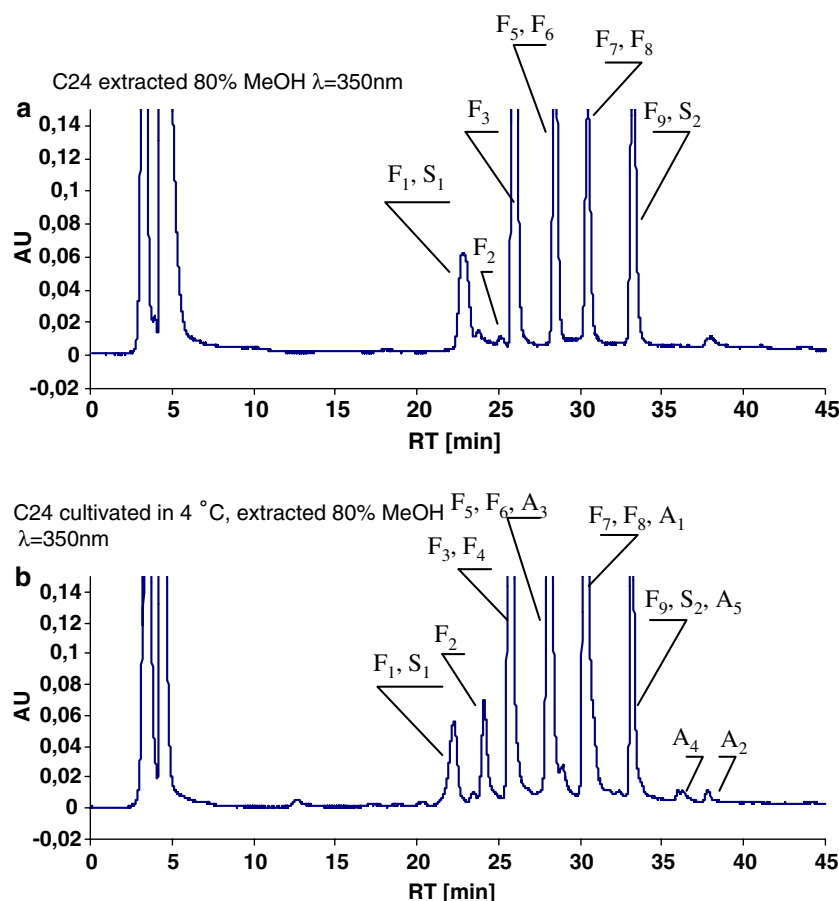


Figure 5. LC/UV chromatograms registered at $\lambda = 350$ nm (compound numbers corresponding to table 1): (a) 80% MeOH extract from leaves of C24 wild type grown in normal temperature, 80% MeOH extract from leaves of C24 wild type grown in 4 °C for 5 days.

3.2.3. LC/MSⁿ detection

Using LC/MSⁿ it was possible to recognize and identify consecutive flavonoid conjugates eluted from the LC column when analyses were performed in CID MSⁿ mode with the triple quadrupole or ion trap analyzer. Product ions derived from protonated $[M+H]^+$ or deprotonated $[M-H]^-$ molecule ions gave sufficient information to suggest the aglycone identity as well as the glycosylation pattern (figures 1–4). However, for the final identification of compounds it was necessary to run product ion spectra on aglycone fragment ions – Y_0^+ , these spectra were compared with those registered for standards of the aglycones (quercetin, kaempferol and isorhamnetin). It should be kept in mind that during the analyses performed in negative ion mode, CID experiments of $[M-H]^-$ ions lead to aglycone ions of anion (Y_0^-) or anion radical ($Y_0^{\bullet-}$) type that registered in the product ion spectra, which is also the case for the first order mass spectra. Further collision induced fragmentation of both ion types (anion and anion radical) is slightly different and a careful examination of registered mass spectra should comply with information regarding energies applied in the ion source as well as in the analyzer during the CID (March et al., 2004).

Sugar moieties in different numbers (two to four rings) and sizes (glucose and rhamnose) are attached to the aglycones of *A. thaliana* flavonoid conjugates. The structures of the compounds analyzed could be proposed on the basis of m/z values of protonated $[M+H]^+$ and deprotonated $[M-H]^-$ molecule ions and fragment ions of $Y_n^{+/-}$ type created after cleavage of glycosidic bonds between the sugar or sugars and the aglycone. The glycosylation pattern on the aglycone moiety could be proposed after the analysis of fragmentation pathways of $[M+H]^+$ and $[M+Na]^+$ or $[M-H]^-$ ions (figures 1 – 4 and 7, 8). The product ion spectra registered after collisions of $[M+H]^+$ or $[M-H]^-$ ions in the positive or negative ion mode, respectively, are not sufficient to provide a conclusive information about the pattern of sugar linkages. However, some conclusions concerning the glycosylation pattern may be obtained.

3.2.4. Identification of flavonol conjugates

We were able to identify nine flavonol conjugates with different glycosylation patterns. The product ion spectra of kaempferol 3-*O*-glucosyl-glucoside 7-*O*-rhamnoside registered in positive mode suggest the elimination of one glucose, which is substituted as diglucoside at C-3

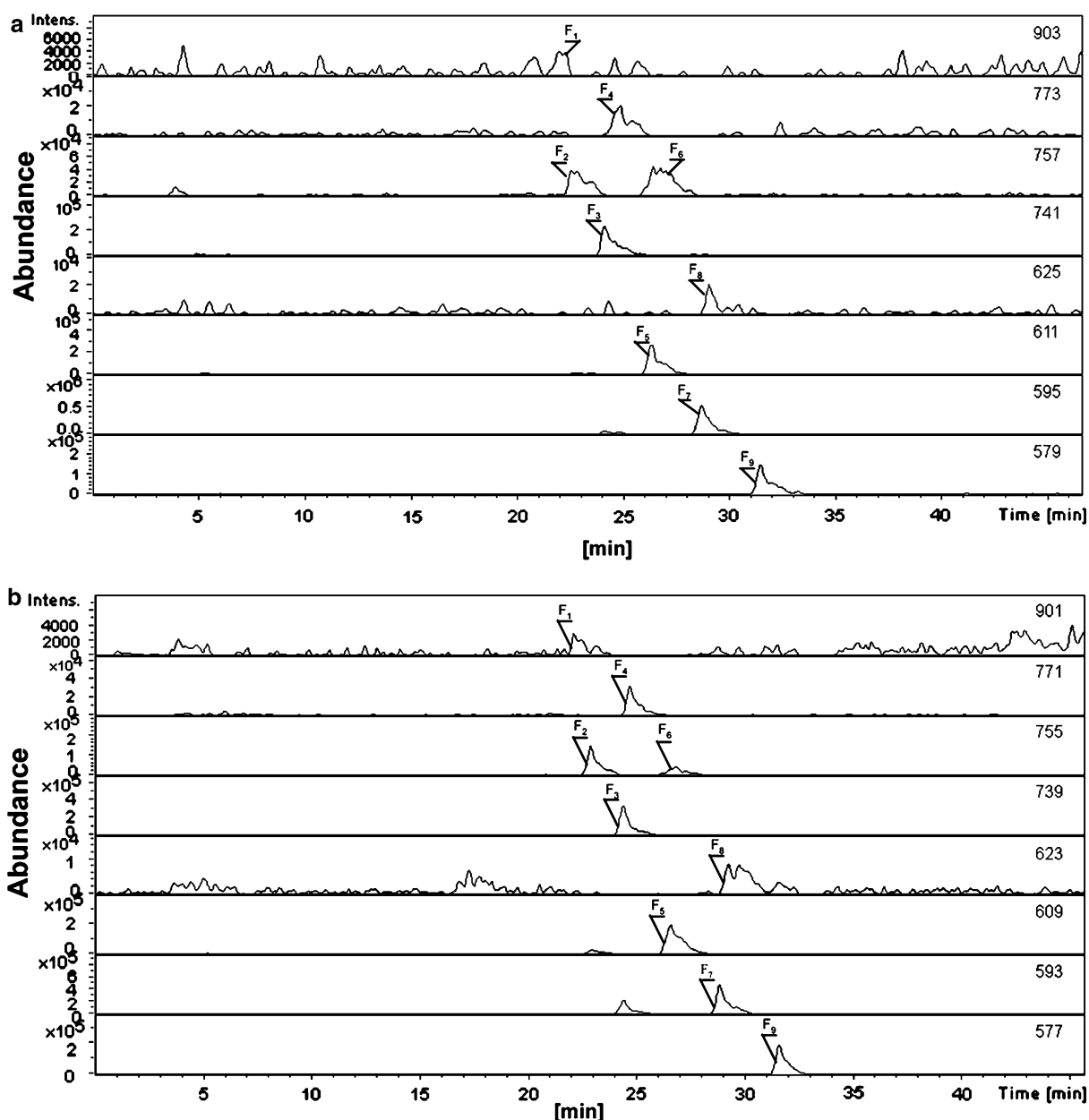


Figure 6. Single ion chromatograms of leaves extract obtained from *A. thaliana* plants (ecotype C24) with suppressed transcription factor gene expression grown in 4 °C for 5 days, registered with ion trap analyzer: (a) Protonated molecules $[M + H]^+$ of flavonol conjugates after CID MSⁿ analysis, (b) deprotonated molecules $[M - H]^-$ of flavonol conjugates after CID MSⁿ analysis, (c) anthocyanin glycosides after CID MSⁿ analysis in positive ion mode, (d) deprotonated molecules $[M - H]^-$ of sinapoyl acid esters.

hydroxyl (figure 1). The mass spectrum of kaempferol 3-*O*-rhamnosyl-glucoside 7-*O*-rhamnoside shows that the first reaction during CID is the elimination of the rhamnose molecule, it may therefore be concluded that it is a part of the rhamnosyl-glucoside moiety attached to the C-3 hydroxyl (figure 2). However, a small fragment ion at 579 *m/z* was also observed, indicating that the rupture of the glucose moiety is also possible after an internal rearrangement of sugars in the disaccharide moiety. The mechanism for this type of sugar cleavage in flavonoid disaccharides was proposed and proven by Ma *et al.* (2000). In the negative mode mass spectra the

elimination of the rhamnose moiety was observed, this fragmentation probably occurred after the elimination of the sugar ring substituted at C-7 hydroxyl (figures 1b, 2b). In contrast, in the positive mass spectra of kaempferol 3-*O*-glucoside 7-*O*-rhamnoside and isorhamnetin 3-*O*-glucoside 7-*O*-rhamnoside the rupture of glucose from the C-3 hydroxyl was noted in the first step of fragmentation (figure 3a, c). Moreover, the elimination of sugars linked to both hydroxyls (C-3 and C-7) was observed in the first step of $[M - H]^-$ ion fragmentation in the negative mode product ion spectra of diglycosides (figure 3b, d). In *A. thaliana* there is only

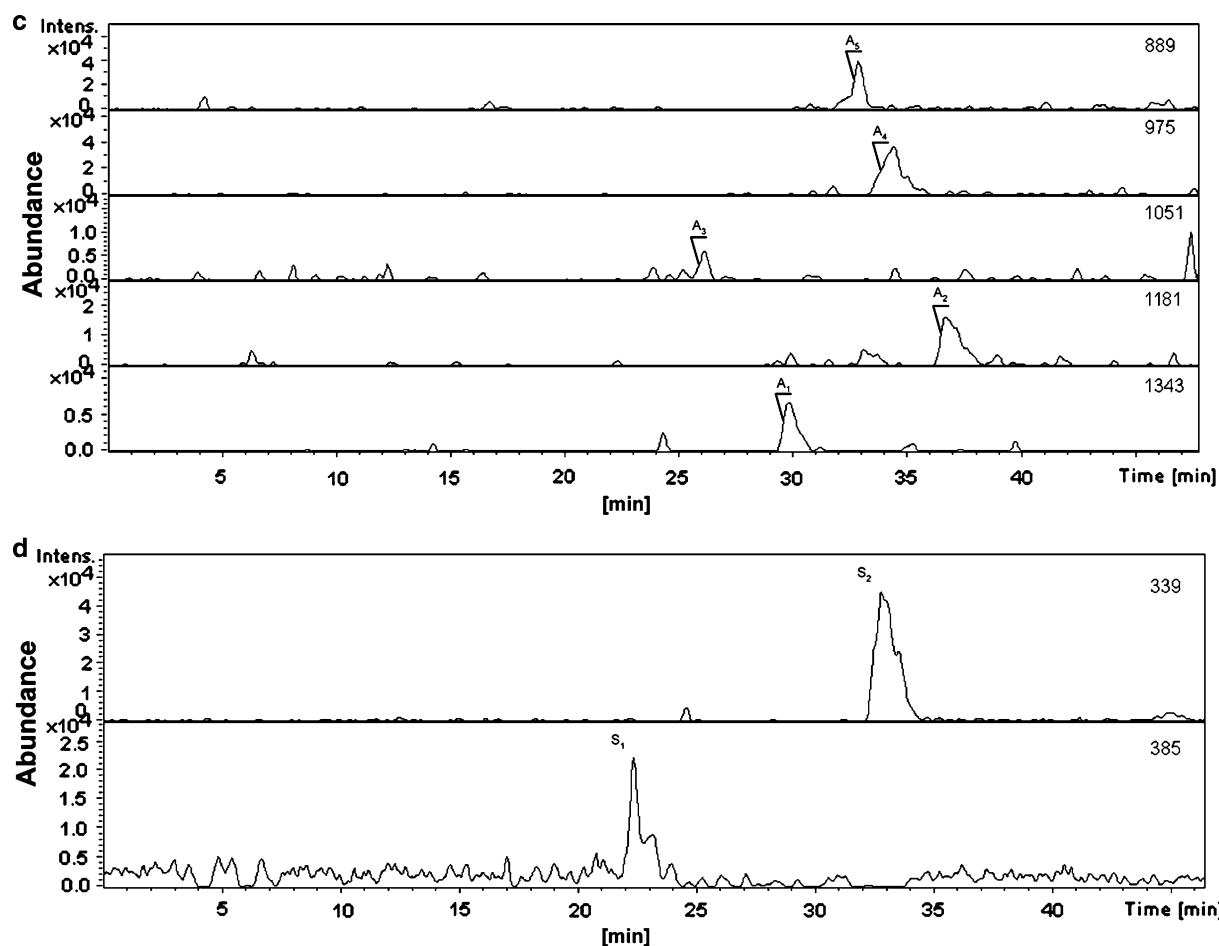


Figure 6. Continued.

one class of flavonoids, these are flavonol conjugates (quercetin, kaempferol and isorhamnetin) and sugars are linked to C-3 and C-7 hydroxyls. Accordingly, some conclusions may be drawn from the registered mass spectra of flavonol glycosides (F1–F9) about the sequence of sugar ring cleavages observed in the positive and negative ion spectra. However, especially valuable for the elucidation of sugar substitution patterns were the CID MS/MS product ion spectra registered for $[M + Na]^+$ ions of flavonoid glycosides. In this case, the localization of the positive charge on the fragment ions took place on the sugar moiety, and the aglycone (kaempferol or quercetin) was cleaved as a neutral molecule. During the fragmentation of the $[M + Na]^+$ ions the sodium cation was exclusively localized on sugar or sugars cleaved from the C-3 hydroxyl group of the flavonol molecule, this fragmentation pathway was registered in the product ion spectra of all the quercetin and kaempferol glycosides studied. As a result of our studies on the *Arabidopsis* C24 ecotype we demonstrated the presence of kaempferol 3-*O*-rhamnosyl-glucosyl-glucoside 7-*O*-rhamnoside. This is the first report of finding flavonol with four sugars bound to an aglycone in *A. thaliana*. In the CID spectrum of kaempferol

tetraglucoside (F1) a fragment ion at 493 m/z composed of three sugars rings was observed (figure 4a), but no information regarding the pattern of sugar linkages in the triglucoside may be obtained from the spectra. We also identified isorhamnetin 3-*O*-glucoside 7-*O*-rhamnoside, the aglycone structure of this compound was confirmed after comparing CID spectra with those obtained for isorhamnetin standard (data not presented).

3.2.5. Identification of anthocyanins and sinapate esters

Five anthocyanins could be identified on the basis of the registered CID mass spectra of M^+ ions. The collision induced fragmentation led to fragments created after elimination of substituents at C-5 and C-3 hydroxyl groups. Malonyl-glucose was always eliminated from C-5 hydroxyl of M^+ ions and the rupture of the substituent at C-3 carbon atom was also observed, the elimination of neutral molecules at this side occurred in one or two steps (figure 7). However, when leaves were extracted with methanol at neutral pH, these compounds could only be detected in samples derived from the wild type plants and from the transgenic line grown at low temperature for 5 days (table 1). To achieve

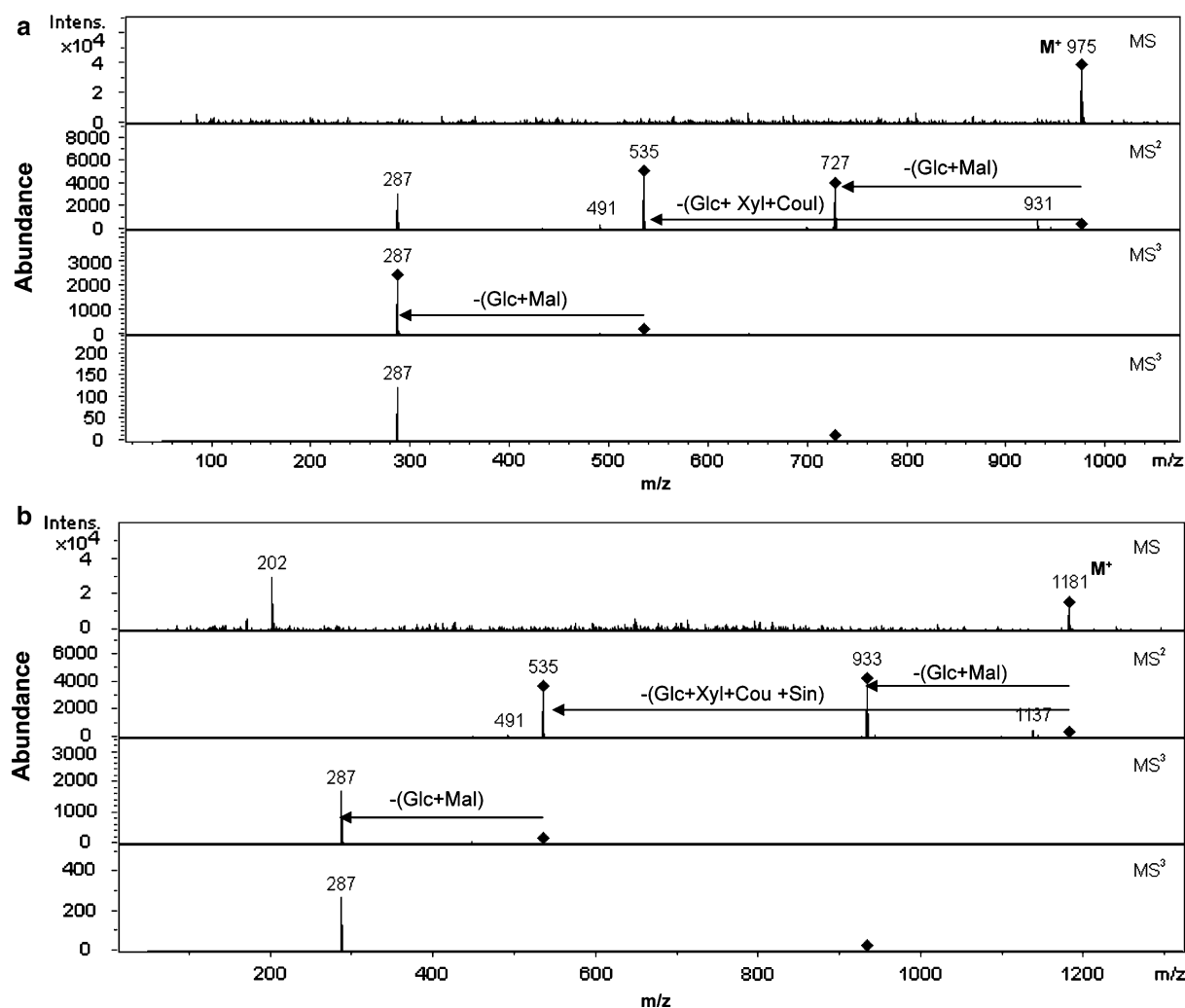


Figure 7. CID mass spectra registered with ion trap instrument: (a) cyanidin 3-*O*-{2''-*O*-(xylosyl) 6''-*O*-[*p*-*O*-(glucosyl)-coumaroyl]-glucoside} 5-*O*-glucoside MW = 975, (b) cyanidin 3-*O*-[2''-*O*-(6'''-*O*-(sinapoyl) xylosyl) 6''-*O*-(*p*-coumaroyl) glucoside] 5-*O*-malonyl-glucoside MW = 1181.

efficient extraction of anthocyanins from *A. thaliana* leaves it was necessary to use an acidified solvent. In our studies we compared the efficiency of phenolic compound extraction using 80% methanol and the same solvent acidified to pH = 3. LC/MS profiling of the target compounds demonstrated that the anthocyanin yield was improved in the acidic extraction medium, whereas the amount of flavonol glycosides was not influenced by a low pH of the solvent used for the extraction (table 1). Concentrations of sinapic acid esters (S1, S2) in the acidic extract were also not affected. Sinapoyl glucose and sinapoyl malate were identified but their identification was much easier when the LC/MS analyses were run in the negative ion mode due to the improved signal to noise ratio compared to the positive ion mode. Deprotonated molecule ions $[M-H]^-$ at 385 and 339 *m/z*, respectively, were observed in the mass spectra and elimination of the glucose moiety or of malic acid after the cleavage of the ester bond occurred. Characteristic fragment ions at

223 *m/z* corresponding to the deprotonated molecule of sinapic acid were registered in the product ion spectra of both compounds, however the intensity of this ion is very much dependent of cone voltage and CID energy applied (figure 8). Further fragmentation of this ion at 223 *m/z* in CID experiments was characteristic for sinapic acid (data not presented). It was not possible to establish the exact placement of the ester bond between the acid and sugar moieties of sinapoyl glucose on the basis of the registered CID mass spectra.

3.3. General remarks on the suitability of tandem mass spectrometry for flavonoid conjugate profiling in *Arabidopsis*

Numerous papers have been published on applications of mass spectrometry in structural analysis of flavonoid conjugates and utilization of LC/MS methods in profiling flavonoid glycosides isolated from plant material (Stobiecki 2000; de Rijke *et al.*, 2003; Cuyckens

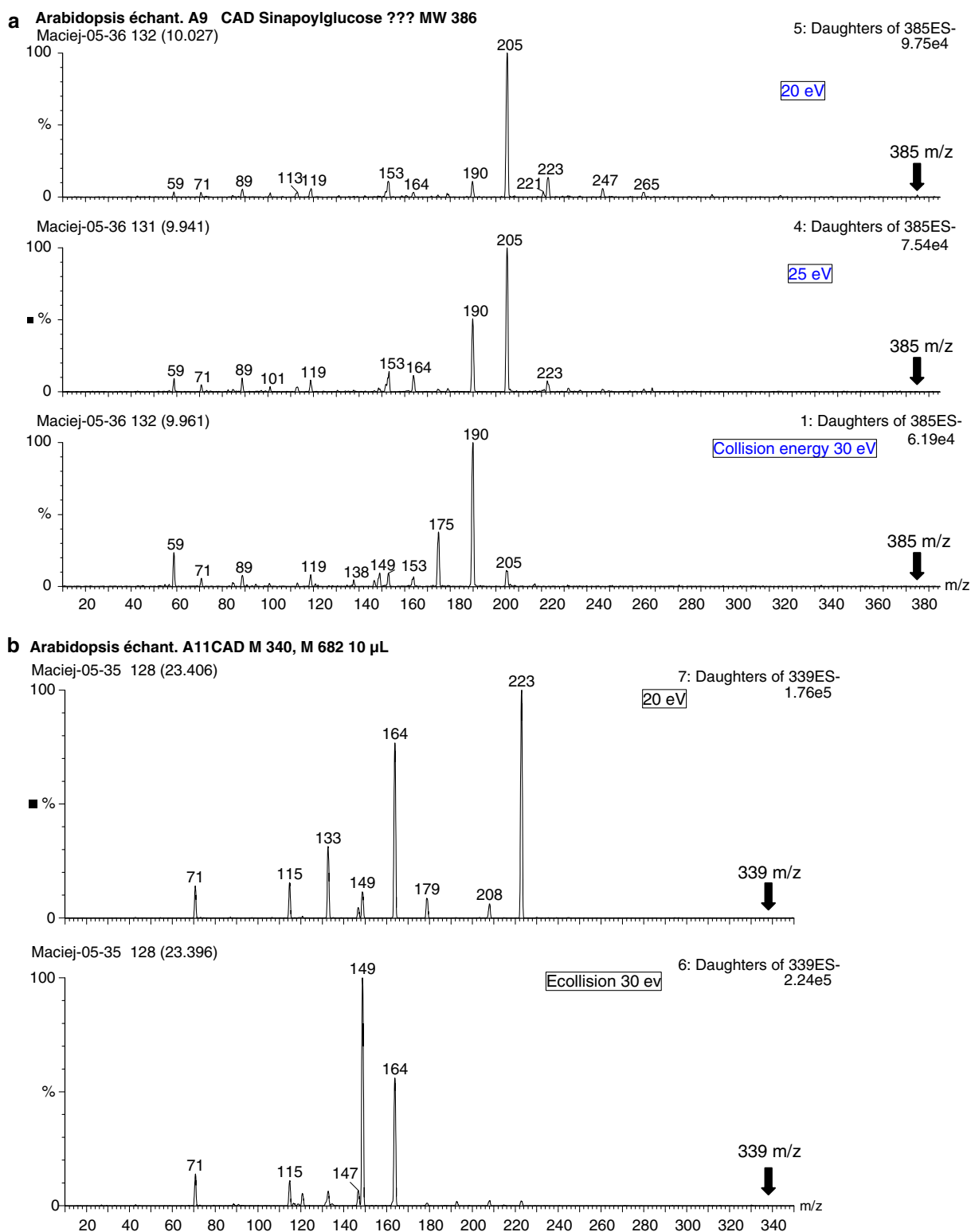


Figure 8. Identification of sinapic acid esters in extracts from *A. thaliana* leaves: (a) product ion spectra of $[M - H]^-$ ion of sinapoyl glucose at 385 m/z (cone voltage 25 V) and collision energy 20, 25 and 30 eV respectively, registered with triple quadrupole analyzer, (b) product ion spectra of $[M - H]^-$ ion of sinapoyl malate at 339 m/z (cone voltage 25 V), and 20 or 30 eV collision energy respectively, registered with triple quadrupole analyzer, (c) product ion MS^2 spectra of $[M - H]^-$ ion of sinapoyl glucose at 385 m/z registered with ion trap analyzer, (d) product ion MS^2 spectra of $[M - H]^-$ ion of sinapoyl malate at 339 m/z registered with ion trap analyzer.

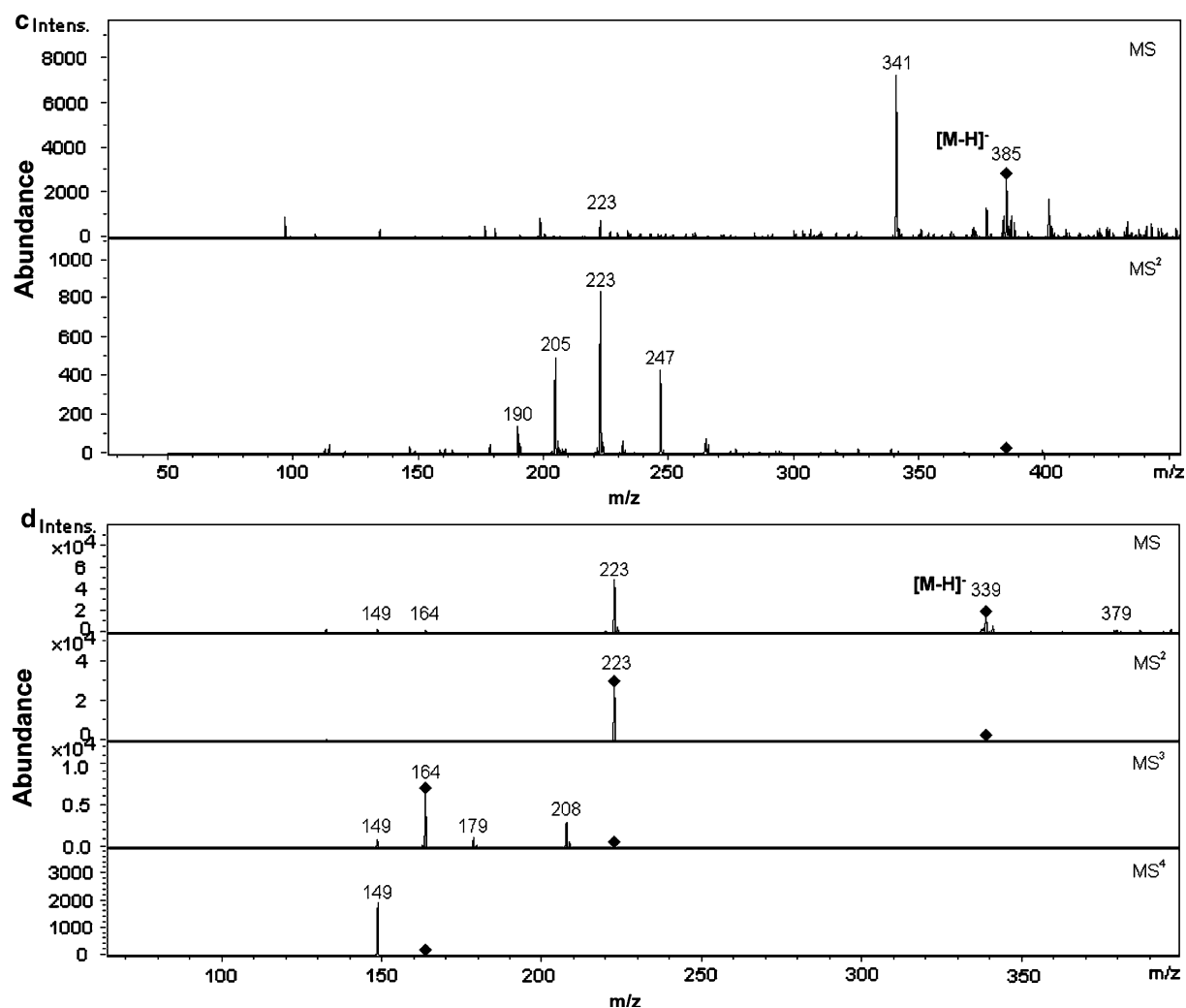


Figure 8. Continued.

and Clayes, 2004; Prasain *et al.*, 2004; Lee *et al.*, 2005; Stobiecki and Kachlicki, 2006). Only few papers were directed towards the profiling of different classes of flavonoids extracted from *A. thaliana* tissues (shoots, roots and seeds; Pelletier *et al.*, 1999; von Roepenack-Lahaye *et al.*, 2004; Le Gall *et al.*, 2005; Kliebenstein *et al.*, 2005; Pourcel *et al.*, 2005; Tohge *et al.*, 2005; Routaboul *et al.*, 2006). From the published data it is clear that substantial qualitative and quantitative differences in flavonoid composition exist among *A. thaliana* genotypes in seeds (Pourcel *et al.*, 2005; Routaboul *et al.*, 2006) and green tissues (Tohge *et al.*, 2005). Proanthocyanidins with different degrees of polymerization of flavan-3-ol units as well as variable amounts of individual flavonol aglycones have been identified in seeds. Also, the amounts of these compounds in seeds were different from those observed in vegetative tissues, in particular high levels of isorhamnetin glycosides were found in seeds. Some differences were also observed in the composition of flavonoid aglycones present in leaves of the ecotypes most often used for molecular-physio-

logical studies. However, in all cases kaempferol glycosides were the main components in leaf extracts. Modifications induced by genetic engineering or mutations can also have pronounced effects on flavonoid conjugate composition, often affecting the relative amounts of the individual compounds.

In functional genomic studies that analyze the role of genes involved in the synthesis of flavonoids a careful qualitative and quantitative analysis of the target compounds is necessary. It should always be kept in mind that numerous isomeric or homologous phenolic secondary metabolites present in plant tissues may have very close chromatographic properties and UV absorbance spectra. Due to these similarities they may be not properly resolved and quantified in LC/UV systems. Similarly, LC/MS systems equipped with atmospheric pressure ionization (ESI or APCI) or the use of a single analyzer system provide only information about the molecular masses of the conjugates and the sizes of the aglycones, but details of the chemical structures of the compounds are not revealed. Liquid chromatography

combined with electrospray ionization mass spectrometry capable to perform collision induced dissociation experiments (LC/ESI/MS/MS) is the method of choice permitting an unambiguous identification and differentiation of numerous secondary metabolites. However, for an efficient LC/MS analysis of such samples it is useful to perform targeted compound analysis based on the selection of m/z values of ions subjected to the CID experiments. This strategy allows to monitor the highest possible number of compounds of interest in the analyzed samples despite large differences in their quantities. In addition, the monitoring of protonated or deprotonated molecule ions provides valuable structural information. Also CID experiments performed on sodiated $[M + Na]^+$ ions provide information about the sugar substitution patterns on the aglycone. According to the data presented here the application of mass spectrometry in combination with CID systems allows the acquisition of reliable information about phenolic secondary metabolites and the discrimination of flavonoid conjugate isomers or homologues in *Arabidopsis* wild type and transgenic plants.

For the unambiguous identification of flavonoid glycoconjugates it is necessary to perform analyzes in both positive and negative ion modes. The most important advantage of negative ions is their higher stability. In the case of flavonoid conjugates there are many reports demonstrating that this property leads to a better signal to noise ratio in the analyzed spectra. However, the interpretation of fragmentation pathways of flavonoid conjugates on the basis of negative ions mass spectra is more difficult than in the case of positive ion mode (Hvattum and Ekeberg, 2003; March *et al.*, 2004; Ablajan *et al.*, 2006). Negative ion analysis is especially important when sinapic acid esters are also analyzed in the studied samples, however, in the case of anthocyanins which naturally have positively charged molecules, the application of the positive ion mode is necessary.

The method of phenolic secondary metabolite extraction from plant tissues also plays a very important role. Acidification of the solvent increases the yield of anthocyanins, whereas the yield of flavonol glycosides in these conditions is not affected compared to the normal methanol extraction protocol. However, when concentrations of anthocyanins in *A. thaliana* tissues increased as a result of the growth conditions (low temperature) it is possible to extract these compounds even with neutral 80% methanol. Comparison of our results with these of Tohge *et al.* (2005) demonstrate that the anthocyanin composition is quantitatively different between wild type plants of ecotypes C24 and Columbia-0, also the numbers of the recognized compounds are different in both ecotypes.

In *A. thaliana* thousands of primary and secondary metabolites exist and co-elution of two or more compounds from an LC column is frequently observed. Co-elution of different flavonols and anthocyanins was

observed in the chromatographic conditions (LC column and gradient) applied in our experiments (figures 5 and 6). Additional analytical problems arose due to the differences in the concentrations of the target metabolites. The abundance of kaempferol glycosides was in many cases at least one order of magnitude higher than quantities observed for derivatives of quercetin, isorhamnetin and cyanidine (table 1). In such a case a targeted compound analysis is necessary to allow the identification of flavonoid conjugates and tandem mass spectrometric techniques have to be applied. Intensities of protonated or deprotonated molecule ions registered for the compounds studied here in the first order mass spectra often are not of the highest intensity, so during a non-targeted analysis the CID MS/MS experiments are not performed on the desired ions originating from phenolic secondary metabolites. In this situation CID spectra for some of the interesting compounds may not be recorded. For the above mentioned reasons ions for CID MS/MS experiments have to be chosen before analysis (see figure 4d, e). In addition to $[M + H]^+$ or $[M - H]^-$ ions also aglycone Y_0^+ or Y_0^- type ions should be fragmented to confirm aglycone structure. This approach permits almost unambiguous structural characterization of flavonoid glycosides and sinapoyl esters present in the extracts obtained from *A. thaliana* plant tissues.

Additional information about the glycosylation pattern of an aglycone may be obtained after the analysis of the CID MS/MS spectra of sodiated molecule ions $[M + Na]^+$ (figure 4a). In the mass spectra fragment ions are created in which the sodium cation is specifically attached to the trisaccharide substituted to C-3 hydroxyl of the flavonol molecule, the same ion type is also observed in the case of di- or monosaccharides. The sodiated saccharide ions are created after cleavage of neutral molecules of an aglycone and sugar attached to C-7 hydroxyl. A similar approach was used by Cuyckens and Claeys (2005), who analyzed the CID MS/MS spectra of deprotonated and sodiated ions of flavonoid glycosides. Attempts to apply different metal complexes with flavonoid glycosides for structural analysis of the glycosylation pattern were also reported (Zhang and Broadbelt, 2005; Zhang *et al.*, 2005).

The profiling data obtained for phenolic secondary metabolites on triple quadrupole and ion trap analyzers applied in LC/MS systems demonstrated that the same structural information about the compounds studied can be obtained with both instruments. Collision induced dissociation of protonated and deprotonated molecule ions $[M + H]^+$ and $[M - H]^-$ allows conclusions about the glycosylation patterns on an aglycone as further fragmentation of flavonoid ion ($Y_0^{+/-}$) provides information about its structure. However, when an ion trap analyzer is used aglycone ions of flavonoid glycosides are fragmented in the second or even fourth step of the CID experiment and intensities of product ions are sometimes

very low. Therefore, in the case of minor components present in the *A. thaliana* leaf extracts the registered spectra did not give full information about the structures. The quality of the CID spectra of aglycone ions is strongly dependent on the quantities of the studied compounds in the sample. However, it should be kept in mind that optimization of key parameters of the ion source optics and CID in the IT mass spectrometer gives product ion spectra in many cases comparable with those obtained with triple quadrupole instruments. Moreover, the possibility of obtaining sequential MSⁿ CID gives very useful structural information on key product ions.

In general, in the product ion spectra of flavonoid glycosides obtained in the positive mode one can expect the presence of all Y_n⁺ type ions created after fragmentation of [M + H]⁺ ions due to the possible cleavage of all glycosidic bonds. However, in the negative ion spectra Y_n⁻ type ions are not systematically detected (Kerhoas *et al.*, accepted). According to the whole set of data obtained from the characterized structures of the present study, the following conclusions may also be drawn: i) in the CID spectra of [M + H]⁺ ions the first sugar or saccharide residue to be eliminated originates from the C-3 position. This regiospecific process occurs in all 3,7-*O*-glycosylated flavonols examined. ii) the fragmentation of the [M - H]⁻ ions proceeds differently starting with a preferential loss of the sugar unit at C-7. However, in the case of the 3,7-*O*-diglycosylated flavonols analyzed with a triple quadrupole instrument, the elimination of the first sugar occurs competitively at C-7 and C-3 whereas the elimination of the sugar unit at C-7 remains favored with the ion trap. This different behavior between both instruments is likely due to the higher energy deposited on ions during the CID process when using triple quadrupole analyzers. This hypothesis may be inferred from the known structures of some *A. thaliana* flavonol tri- and diglycosides characterized using different physico-chemical methods (Veit and Pauli, 1999; Bloor and Abrahams, 2002).

Knowledge of the fragmentation pathways of the flavonol tetra-, tri- and diglycosides during the CID experiments performed in controlled conditions allows for more precise identification of the studied compounds. Tohge *et al.* (2005) have shown that 11 anthocyanin and 6 flavonol derivatives may be detected in different *Arabidopsis* materials on the basis of MS/MS experiments. However, in wild type plants of different ecotypes some structural differences between the flavonol glycosides may be observed, one can also expect differences in concentration of the compounds.

4. Conclusions

We demonstrated here that at least 16 different flavonoid derivatives, anthocyanins and sinapic acid esters are present in leaves of *A. thaliana*, ecotype C24.

Additional compounds may be observed in other ecotypes and transformed lines. These compounds have very similar chromatographic properties and may not be distinguished using analytical approaches based on HPLC/UV, therefore the application of LC/MS for their profiling is necessary. On the basis of the results presented here we can conclude, that the mass spectra have to be registered with controlled settings of the ionization potential in the ion source of the mass spectrometer and the ionization energy applied in the collision chamber. The product ion spectra of protonated or deprotonated molecules ([M + H]⁺ or [M - H]⁻) and aglycone ions ⁿY₀^{+/-} should be included for characterization of individual compounds. Using LC/MS and/or LC/MS-MS in uniform protocols will allow the comparison of flavonoid conjugate profiles obtained in various laboratories. Our data indicate that acidified methanol should be used for the extraction and identification of anthocyanins in *A. thaliana* with an acceptable sensitivity. The application of normal methanol solution for extraction of these compounds may result in a low reproducibility of quantitative analyses.

Acknowledgments

We greatly thank Matthew Hannah for improving our English, and Dr. Karin Koehl and her colleagues from the Green Team for expert plant care. Both are at the MPI of Molecular Plant Physiology, Golm. Acknowledgement of financial support: Aleksandra Skirycz gratefully acknowledges the Ernst Schering Foundation, Berlin, for providing a doctoral fellowship. Additional financial support was provided through the International PhD Programme 'Integrative Plant Science' (IPP-IPS) funded by the DAAD (Deutscher Akademischer Austauschdienst) and the DFG (Deutsche Forschungsgemeinschaft) under No. DAAD Az. D/04/01336, by the Interdisciplinary Research Centre 'Advanced Protein Technologies' (IZ-APT) of the University of Potsdam, and by the Fonds der Chemischen Industrie (No. 0164389).

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