

GC-MS and MALDI-TOF MS Profiling of Sucrose Esters from *Nicotiana tabacum* and *N. rustica*

Łukasz P. Haliński* and Piotr Stepnowski

Department of Environmental Analysis, Institute for Environmental and Human Health Protection, Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80–952 Gdańsk, Poland. Fax: (+48 58) 523 54 72. E-mail: lukasz@chem.univ.gda.pl

* Author for correspondence and reprint requests

Z. Naturforsch. **68c**, 210–222 (2013); received May 5, 2012/March 20, 2013

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) has been applied for the first time to the analysis of the sucrose esters from the surface of *Nicotiana* L. leaves. The profiles obtained for the model plant *N. tabacum* were similar to those from the gas chromatography-flame ionization detector (GC-FID) analysis. The most reproducible results were obtained using a dihydroxybenzoic acid (DHB) matrix. The main advantage of this method is that crude plant extracts can be analysed without sample clean-up. GC-MS analysis of Aztec tobacco (*N. rustica*) extracts revealed the presence of three types of sucrose esters. All identified compounds had three C₄–C₈ acyl chains substituting the glucose moiety, while the fructose part of the molecule was substituted with 0, 1, or 2 acetyl groups. MALDI-TOF MS analysis of the sucrose ester fraction revealed the presence of compounds not eluting from a GC column. Combining the data from both GC-MS and MALDI-TOF MS experiments, we obtained a full sucrose ester profile, which is based on the molecular weight of the compounds and on the number of acyl chains in the molecule.

Key words: *Nicotiana* L., MALDI-TOF MS, Sucrose Esters

Introduction

The aerial parts of many solanaceous plants are covered by a glutinous layer of glucose or sucrose esters, respectively. Both surface sucrose esters (SEs) and glucose esters (GEs) have been reported for a number of *Nicotiana* (Arrendale *et al.*, 1990; Matsuzaki *et al.*, 1989; Severson *et al.*, 1984, 1985), *Solanum* (King *et al.*, 1990, 1993; Neal *et al.*, 1990), *Petunia* (Chortyk *et al.*, 1997), and *Datura* (Hare, 2005) species. All tomato species previously placed in the *Lycopersicon* genus will be considered as *Solanum* species according to Peralta *et al.* (2006). The production of surface glycolipids is associated with the presence of certain types of glandular trichomes (King *et al.*, 1987; Simmons and Gurr, 2005). The large structural diversity of plant SEs was summarized by King *et al.* (1993). Compounds with 3 to 5 short-chain acyl groups substituting both the glucose and fructose moieties were found. Sucrose esters from *N. tabacum* leaves were characterized as 2,3,4,6-tetra-O-acylated compounds, with the glucose moiety completely esterified with C₂–C₇ straight-chain and branched acids, respectively

(Severson *et al.*, 1985). Corresponding glucose esters were also identified in some tobacco cultivars.

The presence of sugar esters on the plant surface affects the behaviour of insect herbivores. Sucrose esters secreted by glandular trichomes of *Solanum berthaultii* Hawkes have been shown to deter settling and probing by the green peach aphid *Myzus persicae* (Neal *et al.*, 1990). And according to Hare (2005) glucose esters of *Datura wrightii* Regel reduced the feeding of some native herbivores. But neither of these authors observed any increased mortality of insects that could be associated with the toxicity of the sugar esters. Similar results were obtained by Goffreda *et al.* (1988, 1989) for GEs from glandular trichomes of *Solanum pennellii* Correll, which reduced feeding and survival of the green peach aphid and the potato aphid (*Macrosiphum euphorbiae*). The effect was possibly associated with both the physical entrapment of aphids and the deterrence of aphid settling. Chortyk *et al.* (1997) showed *Petunia* sugar esters to be toxic towards adults of *Bemisia tabaci*; this is one of the few studies clearly revealing differences in the toxicities of compounds with different structures. Tri- and tetra-substituted SEs

were highly toxic to insects, SEs substituted with five acyl groups and tri-acylglucose ester groups were moderately toxic, while tetra-acylglucose esters were almost inactive. The insecticidal properties of some synthetic SEs substituted with C₆–C₁₂ fatty acids were reported by Chortyk *et al.* (1996) and Li *et al.* (2008). At the same time sugar esters, both synthetic and of plant origin, were shown to be relatively non-toxic to a number of predatory insects (Liu and Stansly, 1996; Michaud and McKenzie, 2004). Hence, these compounds may be a good alternative to commonly used pesticides, as they are also non-toxic to mammals.

Gas chromatography (GC) techniques with FID (flame ionization detector) and MS (mass spectrometry) detection have been widely used for the analysis of both GEs and SEs as trimethylsilyl (TMSi) derivatives (Arrendale *et al.*, 1990; Chortyk *et al.*, 1997; Severson *et al.*, 1985). LC (liquid chromatography), including HPLC (high-performance liquid chromatography) (King *et al.*, 1990) and TLC (thin-layer chromatography) (Simonovska *et al.*, 2006), has also been applied. However, as SEs are usually present as mixtures of isomers, and some plant species produce more than one type of these compounds (Arrendale *et al.*, 1990; King *et al.*, 1993), direct chromatographic analysis is often impossible. Thus, fractionation methods, including chromatography on silicic acid (Chortyk *et al.*, 1997) or Sephadex LH-20 (Arrendale *et al.*, 1990; Severson *et al.*, 1985) and TLC (King *et al.*, 1993), are used. Also, the components of cuticular waxes should be removed prior to analysis by, for example, liquid-liquid extraction (Severson *et al.*, 1985), as they co-elute with SEs. The structures are then determined by MS and NMR (nuclear magnetic resonance). Such methods, however, are time-consuming and thus inappropriate for the screening of large numbers of samples. Einolf and Chan (1984) described the application of direct chemical ionization MS (DCI-MS) to *Nicotiana* SEs analysis, but extracts still had to be purified by SPE (solid-phase extraction) before analysis. The LC/ESI-MS-MS (liquid chromatography-electrospray ionization-tandem mass spectrometry) method was also developed by Ding *et al.* (2006), but extracts from green leaves of tobacco were purified and fractionated prior to analysis. To our knowledge, only a single LC/ESI-TOF MS-based method for SEs profiling in crude plant extracts has been described to date (Weinhold and Baldwin, 2011). The successful application of ESI-TOF MS to the

analysis of SEs suggests the usefulness of simpler MS-based methods. The objective of this study was therefore to develop a straightforward procedure for the MALDI-TOF MS (matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry) analysis of SEs in crude plant extracts. The results obtained in this way were compared with those from GC-FID analyses of *N. tabacum* SEs. The possibility of determining both the relative composition and the exact quantity of SEs is discussed. The application of the method to the analysis of the more complex mixture of *N. rustica* sugar esters is also presented.

Experimental

General experimental procedures

Solvents from local distributors (p. a. grade) were used as received except dichloromethane, which was distilled before use. MALDI matrices: dihydroxybenzoic acid (DHB), sinapinic acid (SA), α -cyanohydroxycinnamic acid (CHCA), and 2,4,6-trihydroxyacetophenone (THAP), as well as *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with trimethylchlorosilane (TMCS) (99:1), *n*-docosane, and sucrose monolaurate, were all obtained from Sigma Aldrich (Poznań, Poland).

GC-MS analyses were performed on a Shimadzu QP-2010SE system (Shimadzu, Kyoto, Japan). Helium was used as carrier gas at a flow rate of 1 ml min⁻¹. GC-FID analyses were performed on a Clarus 500 gas chromatograph (Perkin-Elmer Instruments, Waltham, MA, USA), with an RTX-5 capillary column (30 m x 0.25 mm i.d., 0.25 μ m film thickness; Restek, Bellefonte, PA, USA). Argon was used as carrier gas at a flow rate of 1 ml min⁻¹. MALDI-TOF MS analyses were performed on a Biflex III spectrometer (Bruker Daltonics, Bremen, Germany) with an N₂ laser (λ = 337 nm).

Plant material and extraction procedure

Plants – *N. tabacum* L. cv. Książnicki and *N. rustica* L. cv. Machorka Brazylijska – were grown from May to August 2011 in a greenhouse without temperature control (temperature between 22 and 32 °C during the day and between 15 and 20 °C at night; no supplemental lighting was provided). The seeds were obtained from the Institute of Soil Science and Plant Cultivation (Puławy, Poland). Four weeks after germination, the seedlings were transplanted to ca. 8-l pots.

Plants were kept well-watered by irrigating 2–3 times a week (400–600 ml, depending on the weather conditions). SEs were extracted from the leaves of twelve-week-old plants by dipping the leaves (ca. 20 g per sample) in 800 ml dichloromethane for 40 s. Additionally, fruit capsules (ca. 10 g) from sixteen-week-old *N. rustica* plants were extracted using 200 ml of dichloromethane. All extracts were dried with anhydrous sodium sulfate, filtered, and evaporated to ca. 1–2 ml under reduced pressure.

Isolation of sugar esters from the extract

One part of each extract was left untreated for direct GC-MS and MALDI-TOF MS analyses, while the remainder was fractionated by liquid-liquid extraction according to the modified procedure of Severson *et al.* (1985). About half of the extract was partitioned between 25 ml of *n*-hexane and 20 ml of 80% methanol. The *n*-hexane layer was then extracted with 20 ml 80% methanol. A mixture of 13 ml water and 7 ml saturated aqueous potassium chloride was added to the combined aqueous methanol fractions. Sugar esters were extracted twice with 25 ml dichloromethane. The combined extracts were filtered through sodium sulfate, and the solvent removed under reduced pressure.

GC-MS and GC-FID analysis

The sugar esters in both crude extracts and purified fractions were analysed as their TMSi derivatives. After removal of the solvent under a stream of nitrogen, 100 μ l BSTFA + TMCS (99:1) were added, and the sample was heated for 30 min at 90 °C. The quantification of SEs from *N. tabacum* leaves in the purified sugar ester fraction was based on their peak areas from GC-FID analysis, compared to the peak area of the internal standard *n*-docosane, according to the equation:

$$m_S = \frac{m_{IS} \cdot A_S}{A_{IS}},$$

where m_S is the mass of a compound (unknown), m_{IS} is the mass of the internal standard, A_S is the peak area of a compound, and A_{IS} is the peak area of the internal standard. The response factors were not determined due to the lack of commercially available SE standards. The relative composition of the GE fraction in *N. rustica* extracts, as

well as the GE contribution to total GC-eluting sugar esters, was determined using their peak areas from GC-FID analyses. The mean values from three analyses were calculated.

GC-MS analyses of sugar esters were performed using a 30 m x 0.25 mm i.d., 0.1 μ m film thickness, Rxi-5HT capillary column (Restek). The injector and GC-MS interface temperatures were 320 °C, and the column temperature was programmed from 100 to 320 °C at 4 °C min⁻¹, then held at 320 °C for 5 min. Electron-impact ionization (electron energy, 70 eV; ion source temperature, 200 °C) was used. GC-FID analyses were performed using an RTX-5 capillary column (30 m x 0.25 mm i.d., 0.25 μ m film thickness; Restek). The injector and detector temperatures were held at 320 °C. The column temperature programme was as in the GC-MS analysis. In both cases, the split ratio was 1:20, and the injection volume was ca. 1 μ l.

MALDI-TOF MS analysis

Isolated sugar ester fractions were analysed as solutions in methanol. Substances extracted from ca. 1 g of fresh weight (FW) plant material were present in 1 ml of the solution. Crude extracts were dissolved in methanol/dichloromethane (50:50, v/v). A few concentrations were tested, ranging from 1 g to 0.001 g of FW plant material per 1 ml. A further series of samples was analysed with the addition of 0.5 mg of sucrose monolaurate as internal standard. All analyses were performed in triplicate. Samples were mixed with the solution of the matrix in methanol or acetone, applied on the sample support plate, and left to air-dry. Spectra were recorded in the positive ion linear mode, by averaging 160–220 scans. The relative composition of the SE fraction in all extracts was determined using the peak heights of SEs of specified molecular weight, in relation to the sum of peak heights of all SEs detected in a sample.

Results and Discussion

Almost all published procedures for the analysis of SEs from plant surfaces require a multi-step sample clean-up; thus, a one-step procedure for the analysis of crude plant extracts would be of the greatest interest. During our studies of the surface chemistry of solanaceous plants we decided to develop a fast analytical meth-

od for screening SEs in extracts. Although the MALDI-TOF MS technique is commonly used to analyse high molecular weight biomolecules, the analysis of low molecular weight compounds is also possible (Cohen and Gusev, 2002). We chose *N. tabacum* as a model plant because of the simplicity of the SEs fraction synthesized by this species. Compounds of only one type of SE (referred to as SE-I throughout the text) have been identified by most authors and are well characterized. Previous reports have also shown that *N. tabacum* SEs can be easily analysed by GC when purified, so GC can be used as a reference technique.

Identification of N. tabacum sucrose esters by GC-MS

Compounds in the extracts from *N. tabacum* cv. Książnicki were first analysed by GC, without purification, as their TMSi derivatives. The predominance of cuticular hydrocarbons over the SEs fraction, however, made direct analysis unreliable. The SEs were therefore separated from cuticular waxes according to Severson *et al.* (1985), and the SEs fraction was analysed once again. The chromatograms (Fig. 1) showed five partially separated clusters of peaks. GC-MS analysis iden-

tified the fraction components as typical SE-I compounds. Each cluster of peaks represented a number of isomers, probably differing in the position of the acyl chains in the molecule. The mass spectra of the compounds showed the presence of characteristic series of ions at m/z 443, 457, 471, 485, and 499, indicative of a tetra-acyl glucose moiety. The presence of ions at m/z 451, 437, and 361 confirmed the tetra-TMSi fructose moiety, with no acyl groups substituting any of the fructose hydroxy groups. We also recorded a series of ions representing C_2 – C_7 saturated acyl groups. Altogether the mass spectra clearly displayed the presence of well-known *N. tabacum*-type SEs with molecular weights (M_r) of 622, 636, 650, 664, and 678, described earlier by Severson *et al.* (1985).

In addition to these well-characterized compounds, we also identified two SEs bearing one monounsaturated acyl chain. The overall fragmentation of these compounds is similar to that observed for saturated SEs, which suggests the same substitution pattern. The ions of the glucose moiety at m/z 483 and 497, together with the signal of the unsaturated C_6 acyl chain at m/z 97, indicated the presence of SEs similar to those reported earlier by Ding *et al.* (2006).

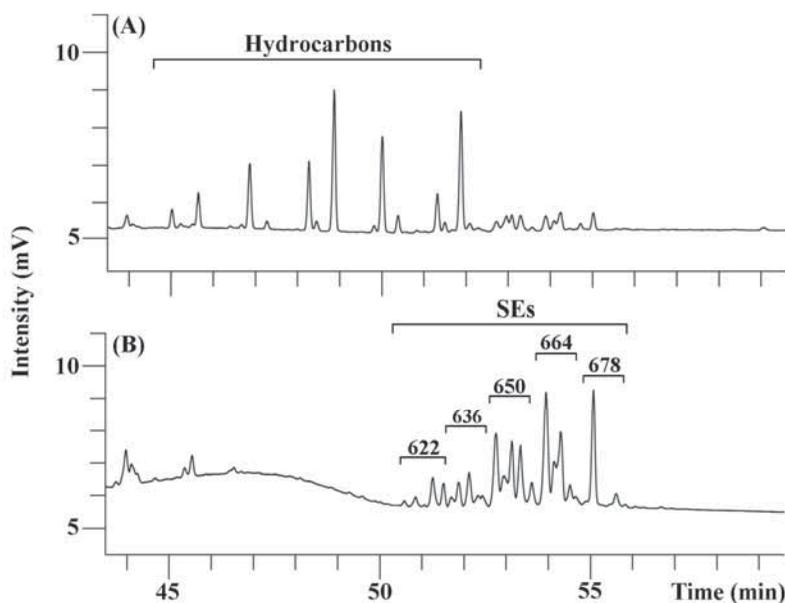


Fig. 1. Partial gas chromatograms of (A) crude extract and (B) sugar ester fraction of *N. tabacum* leaves after trimethylsilylation. Numbers correspond to molecular weights of SE-I-type sucrose esters.

Identification of *N. rustica* sugar esters by GC-MS

There are a few reports suggesting that there are no sugar esters on the surface of the Aztec tobacco (*N. rustica* L.). Simonovska *et al.* (2006) developed a TLC-based method of SEs analysis and did not detect any SEs in a number of wild tobacco species, including *S. rustica*. Pittarelli *et al.* (1993), in turn, found extracts from *N. rustica* plants to be non-toxic to greenhouse whitefly nymphs, whereas extracts from some other tobacco species were moderately or highly toxic. We, on the other hand, while cultivating *N. rustica* cv. Marchorka Brazyljska plants, observed the formation of very large amounts of sticky sugar compounds.

Extracts from the leaves and immature fruit capsules of the plants were obtained, and after purification, the sugar esters fraction was analysed by GC-MS. Some typical chromatograms are shown in Fig. 2. The results show a complex mixture of one (leaves) or two (fruit capsules) groups of GEs, followed by at least two (leaves) or three (fruit capsules) types of SEs. These findings are in contrast to the previously cited reports and suggest a high intraspecific variation in the synthesis of sugar esters by *N. rustica*. This may highlight the importance of certain environmental factors affecting GEs and SEs production.

The mass spectra of the TMSi derivatives of GEs from *N. rustica* leaves gave a series of $[M-15]^+$

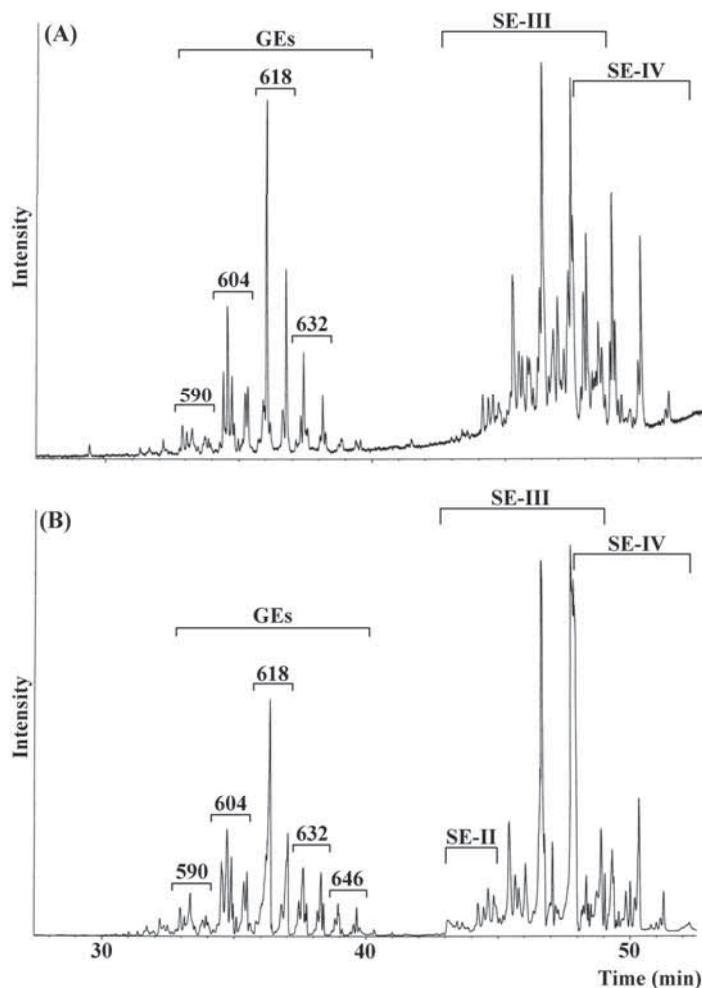


Fig. 2. Total ion current from GC-MS analyses of trimethylsilyl derivatives of the sugar ester fraction from (A) leaves and (B) fruit capsules of *N. rustica*. Numbers correspond to molecular weights of GE-I-type glucose esters.

ions at m/z 575, 589, 603, and 617, indicating the presence of GEs with three acyl groups in the molecule. The signals of C_5 , C_6 , and C_7 acyl groups were observed at m/z 85, 99, and 113, respectively. There were no signals of C_8 and C_9 acyl groups in the spectra, and the total number of carbon atoms in the acyl chains of the GEs detected was 16–19 (based on the molecular weights of the compounds). Hence, these GEs were not like those in the GE-I fraction from *N. glauca* (Severson *et al.*, 1994), where one hydroxy group was esterified with an acetyl group, but rather similar to those described as the GE2 fraction from *Petunia* plants (Chortyk *et al.*, 1997). Those compounds will be referred to as the GE-I fraction. The same GEs were dominant in extracts of the fruit capsules, with an additional compound giving an $[M-15]^+$ signal at m/z 631. In addition, we also detected small amounts of co-eluting GEs of a different type, with $[M-15]^+$ ions at m/z 605, 619, and 633, indicative of a glucose moiety with acyl chains substituting two hydroxy groups (GE-II fraction). The full mass spectra of GE-II compounds were not recorded as their peaks overlapped the signals of the much more abundant GE-I compounds. However, they are probably similar to the GE-II compounds identified in *N. glauca* by Severson *et al.* (1994).

Whereas the SEs fraction in the *N. tabacum* extracts consisted of only one type of compounds, *N. rustica* plants produced a much more complex mixture of those esters. GC-MS analysis of the fruit capsule extracts revealed the presence of three major types of SEs. Compounds from all three types gave similar series of ions at m/z 487, 501, 515, 529, 543, and 557, indicating a glucose moiety substituted with three C_4 – C_7 acyl groups. However, not all of those signals appeared in the spectra of all three types of compounds. The differences between the SEs from the identified groups showed up when the signals due to the fructose moiety were taken into account. The SE-II fraction yielded ions at m/z 437 and 451, indicating a tetra-TMSi fructose moiety. The SE-III fraction was characterized by ions at m/z 407 and 421, which suggests a tri-TMSi fructose moiety with one acetyl group. The presence of the rearrangement ion at m/z 407 indicates, that the acetyl group is on a carbon atom other than 1' (Severson *et al.*, 1994), as the transfer of the TMSi group from the 1' atom of the fructose moiety to the 2' atom is possible. Those two types of SEs

were previously reported in other *Nicotiana* and *Petunia* species (Arrendale *et al.*, 1990; Chortyk *et al.*, 1997). The SE-IV gave an abundant ion at m/z 391, indicative of a di-TMSi fructose moiety with two acetyl groups. The second acetyl group was probably present on the 1' carbon atom of the fructose moiety, as the rearrangement ion at m/z 377 was not observed. The SE-IV compounds were different from those identified by Severson *et al.* (1994) in *N. glauca*, where the glucose moiety was substituted with two acyl groups. A diacetyl-substituted fructose part of the molecule was also reported for *N. cavicola*, but the additional acyl group was present on the fructose moiety (Ohya *et al.*, 1994). An overview of the chemical structures and diagnostic ions of all SE groups is shown in Fig. 3, and their mass spectra are given in Fig. 4. In addition to types SE-II, SE-III, and SE-IV, trace amounts of the SE-I compounds were detected. The composition of the SEs fraction from *N. rustica* leaves was similar, but SE-II compounds were not found. The impact of some environmental factors on the SEs production and the importance of plant age will have to be studied in detail in the future, as our preliminary studies on the SEs composition of *N. rustica* have suggested the presence of much more abundant SE-I compounds (Haliński and Bieniak, unpublished data), which were almost absent in the current study.

Quantification of sugar esters by GC-FID

As only one type of SEs was identified in the sugar ester fraction of *N. tabacum* and signals attributed to compounds of different molecular weight were not overlapping, the direct quantification of SEs eluting from the gas chromatograph was possible. The purified sugar ester fraction with a known amount of *n*-docosane as internal standard was then analysed. The total content of SEs in fresh *N. tabacum* leaves was determined as ca. 0.11% (1.09 mg g⁻¹ of FW plant material) by GC-FID. The unsaturated SEs could not be quantified by GC, as their signals were hardly separated from the much more intense peaks of the corresponding saturated compounds. The relative composition of the SEs fraction was determined in two different scenarios. The first involved the triple analysis of the same sample, giving only the uncertainty of the technique used, while the second included three single analyses of independent

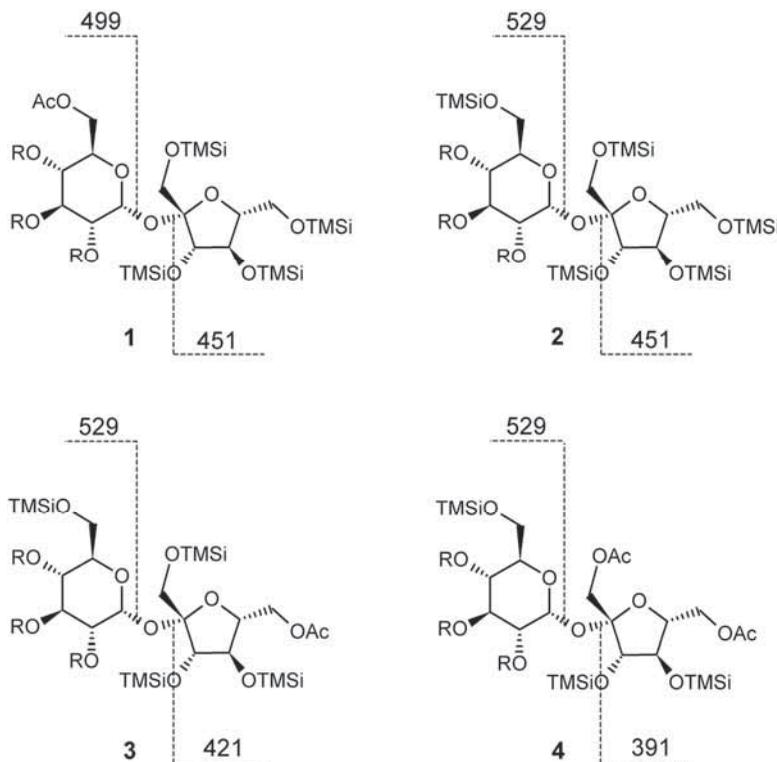


Fig. 3. Examples of structures of sucrose esters from *N. tabacum* (**1**, SE-I type) and *N. rustica* (**2**, SE-II type; **3**, SE-III type; **4**, SE-IV type). Origins of the main diagnostic ions in the GC-MS analysis of the trimethylsilyl derivatives are given ($R = C_6$ acyl group). The position of the acetyl groups was not determined.

samples obtained from three individual plants, also showing between-plant differences in the SEs composition. The results are shown in Fig. 5. The low uncertainty of the technique, measured as relative standard deviation ($RSD \leq 2.5\%$), allowed to display between-plant differences in the SEs profile, which suggest some intraspecific variation of yet unknown origin.

The relative composition of *N. rustica* GEs was also successfully determined by GC-FID analysis. The GE-I and GE-II types could not be distinguished in the extract from the fruit capsules (see Fig. 2 for the order of elution). The contribution of GEs to the total GC-eluting sugar esters was ca. 18.6% in the leaf extracts and 28.2% in the fruit capsule extracts. The relative composition of the fractions is given in Table I. Quantitative chromatographic analysis of *N. rustica* SEs was impossible because the signals of all identified compounds overlapped (see Fig. 2). Also, the pattern of the acyl chains in single compounds was sometimes difficult to determine, as many spectra

were more or less affected by the spectra of adjacent compounds. An exact analysis would require fractionation of the extract in order to obtain pure SEs of a single type.

MALDI-TOF MS analysis of *N. tabacum* sucrose esters

MALDI-TOF MS analyses of *N. tabacum* SEs were performed on the crude plant extracts without any clean-up, at concentrations corresponding to 0.1 g of FW plant material per 1 ml. While the chromatographic analysis of *N. tabacum* SEs resulted in the formation of clusters of peaks due to the presence of a unspecified number of isomers of each compound, the MALDI-TOF MS analysis yielded a simple series of six main peaks at m/z 645, 659, 673, 687, 701, and 715, corresponding to the $[M + 23]^+$ ($[SE + Na]^+$) signals. The interpretation of the results was thus much simpler than after GC analysis, but the information on the presence of different isomers was lost. Detailed

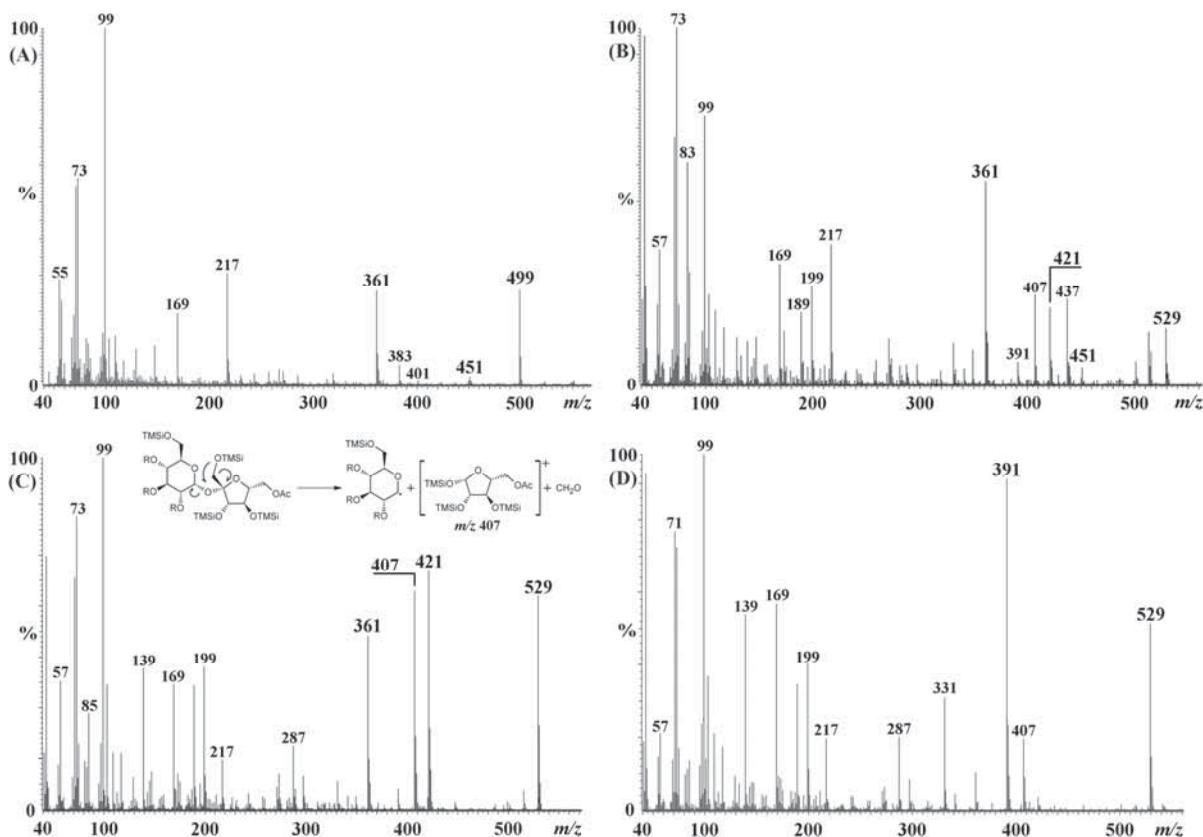


Fig. 4. Mass spectra of trimethylsilyl derivatives of sucrose esters from (A) *N. tabacum* (SE-I type) and (B, C, D) *N. rustica* [SE-II (B), SE-III (C), and SE-IV (D)]. Structures of the compounds are shown in Fig. 3. The origin of the rearrangement ion at m/z 407 in the SE-III type is also given.

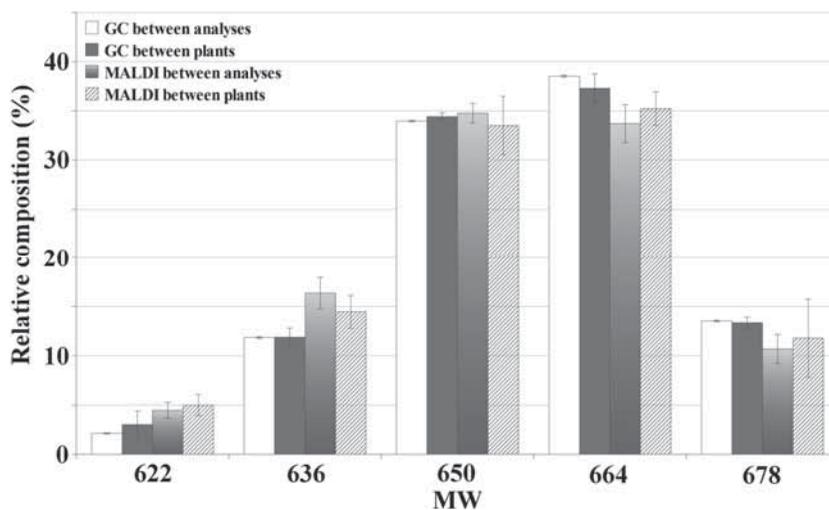


Fig. 5. Comparison of the sucrose ester profiles of *N. tabacum* obtained by GC-FID and MALDI-TOF MS, showing differences in between-plants and between-analyses variation. Bars indicate SD for $n = 3$.

Table I. Relative composition of the *N. rustica* glucose ester fraction (given as % of total GEs). The results are mean values from three analyses \pm SD, based on the peak areas from GC-FID experiments.

M_r	Relative composition (%)	
	Leaves	Fruit capsules
590	12.6 \pm 0.1	6.1 \pm 0.1
604	27.6 \pm 0.9	23.4 \pm 0.1
618+620 ^a	40.3 \pm 0.4	50.8 \pm 0.1
632+634 ^a	14.4 \pm 0.1	16.7 \pm 0.2
646+648 ^a	5.1 \pm 0.3	3.0 \pm 0.2

^a The second value represents compounds of the GE-II type which were detected only in the fruit capsule extracts.

examination of the spectra also revealed signals corresponding to the unsaturated compounds, at m/z 2 units less than for saturated SEs. They were, however, present only in trace amounts. Although all spectra utilized in this study were obtained using a DHB (dihydroxybenzoic acid) matrix, we also tested three other commonly used matrices: CHCA (α -cyanohydroxycinnamic acid), SA (sinapinic acid), and THAP (2,4,6-trihydroxyacetophenone). The DHB matrix produced the most reproducible results, with the highest signal-to-noise (S/N) ratio (Fig. 6). Moreover, the intensities of signals attributed to other substances present in the sample were the lowest among all tested matrices. Similar results, but with slightly lower S/N values, were also obtained with the THAP matrix. The SA matrix gave variable S/N ratios – from very high to extremely low; it also produced many signals in the m/z region of the spectrum where the SEs signals appeared. Moreover, it tended to produce broadened signals, which affected the quantitative analysis, precluding the detection of some compounds (e.g. the unsaturated SEs from *N. tabacum*). Similarly, the CHCA matrix gave no reproducible results, as the S/N ratio varied broadly. The next step was to perform a semi-quantitative analysis of SEs based on peak heights in order to obtain the SEs profiles of the *N. tabacum* extracts. The relative composition of the SEs fraction was determined in two different scenarios, as described in the preceding paragraph. The results (covering only compounds eluting in GC) are shown in Fig. 5, together with the results from the GC-FID analyses. The last compound detected by MALDI-TOF MS (M_r 692) was not detected by GC. Comparison of the results revealed a higher reproducibility of the

GC technique in comparison with MALDI-TOF MS. Uncertainty, measured as standard deviation (SD), was higher with the latter technique, especially for the minor components of the fraction. However, for the main SEs, it gave satisfactory results (RSD \leq 5%). Hence, we can consider MALDI-TOF MS sufficient for SEs profiling. There is a possibility of losing some information on the intraspecific variation in the SEs content, as the error of the MALDI-TOF MS analysis is in some cases as high as the between-plant variation (see compounds with M_r 622, 636, and 664 in Fig. 5). With this technique, however, compounds can be analysed that are not volatile enough to elute from a standard GC capillary column. Finally, we successfully applied MALDI-TOF MS to the analysis of the crude plant extracts, which was impossible with GC.

MALDI-TOF MS analysis of *N. rustica* sucrose esters

The ions attributable to GEs were not observed in the mass spectra, thus GEs must be analysed using standard GC-FID and GC-MS procedures. The application of MALDI-TOF MS to the analysis of SEs from *N. rustica* leaves and fruit capsules yielded additional information on the number of higher-mass compounds not eluting from the GC column. All signals were present as $[M + 23]^+$ ions; the DHB matrix was used in all measurements. A typical spectrum obtained for fruit capsules is shown in Fig. 7. As the MALDI-TOF MS data did not distinguish between the compounds from the different types of SEs, the profiles are based on the molecular weight, which depends on the number of carbon atoms present in the acyl chains. The relative composition of the fraction determined for the crude extracts is shown in Table II, together with an overview of the possible combinations of compounds detected as single peaks in the spectra. The reproducibility of the results was slightly lower than in case of *N. tabacum*, with relative SD values of 5–7% for the major SEs and 3–23% for minor components. Some compounds were excluded based on the results of the GC-MS analyses. For example, we did not detect SE-II compounds with $M_r > 636$, so it is improbable that SEs from this group contributed to the signals on the spectra at $m/z > 659$. At the same time, we did not detect SE-III compounds with $M_r < 636$ and > 706 , or SE-IV compounds with

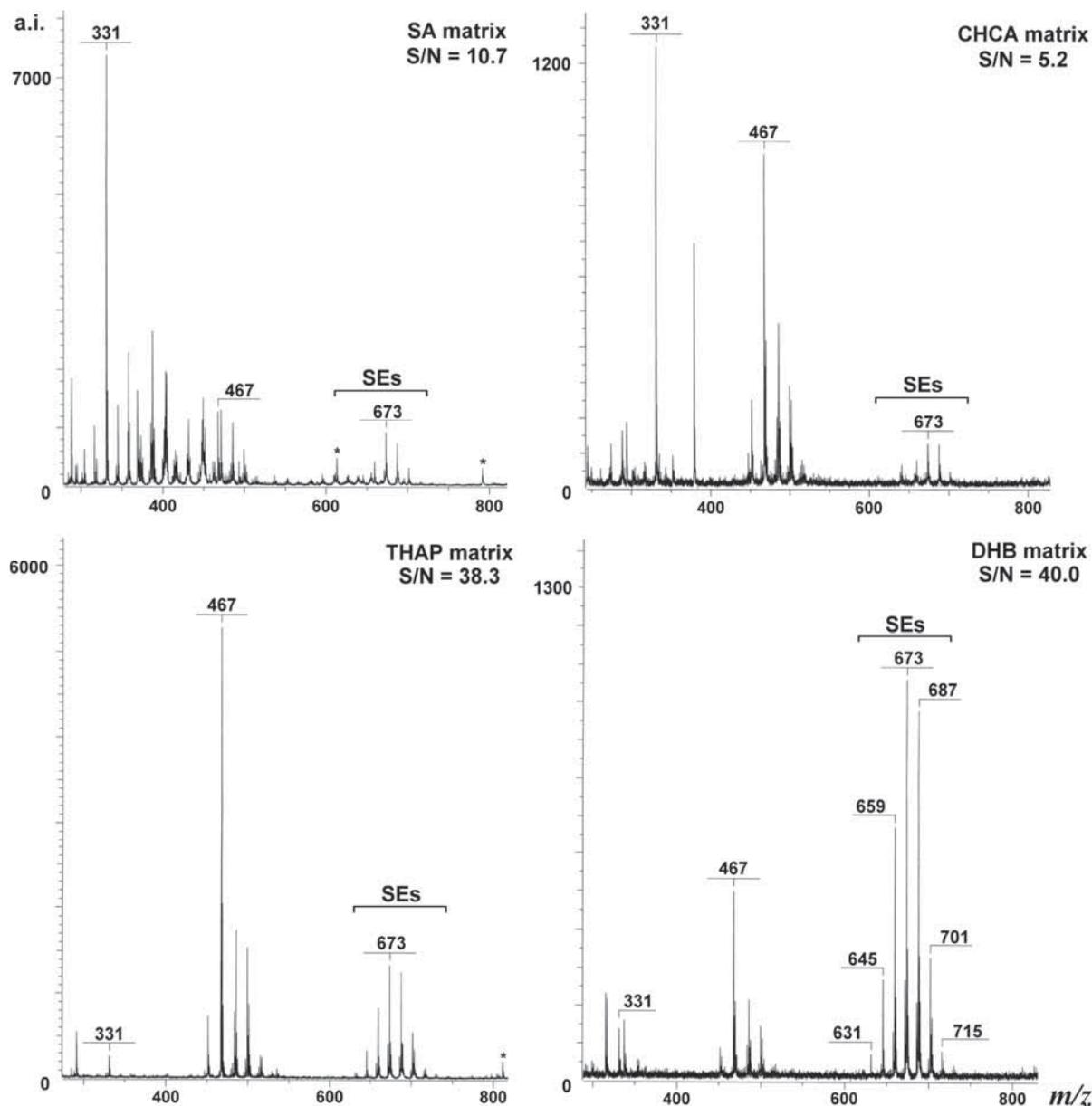


Fig. 6. MALDI-TOF mass spectra of *N. tabacum* sucrose esters, obtained using SA, CHCA, THAP, and DHB matrices. The signal-to-noise ratio (S/N) is given for a main SE in the fraction (m/z 673). On the spectrum obtained with DHB matrix, $[M + 23]^+$ values are given for each signal attributed to sucrose esters.

$M_r < 692$. Thus, SE-III compounds contributed to the signals between m/z 659 and 729, while the SE-IV fraction was represented by the signals at m/z 715 and higher. MALDI-TOF MS profiling of such a complex mixture of SEs thus allows the approximate determination of the relative composition of the fraction, which applies not only to

the molecular weight, but also to the number of acyl groups in the molecule. As shown by Chortyk *et al.* (1997), SE toxicity towards insects is, at least to some extent, a function of the number of acyl chains, which determines the polarity of the whole molecule. Compounds with five acyl groups in a molecule are only moderately toxic, and toxicity

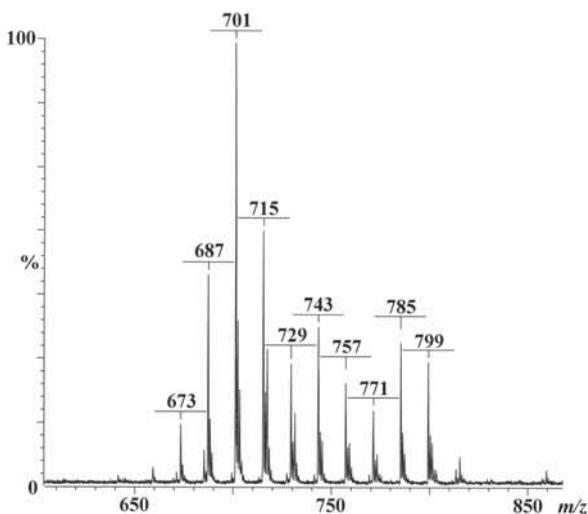


Fig. 7. MALDI-TOF mass spectrum of sucrose esters from *N. rustica* fruit capsules, recorded in DHB matrix; $[M + 23]^+$ values are given for each signal.

increases with decreasing number of acyl chains. Thus, when SEs of many types are produced by the species in question, MALDI-TOF MS analysis of the crude extracts should be accompanied by GC-MS analysis of the sugar ester fraction after removal of the cuticular hydrocarbons. Such a procedure allows profiling to be carried out whilst not ignoring the potential biological activity of

the compounds. Nonetheless, fractionation of the sample into single types of SEs is not necessary to obtain valuable results.

Optimization of MALDI-TOF MS analysis and quantification of sucrose esters

Selection of the MALDI matrix has been described above. During the experiment we also realized the importance of the sample concentration for obtaining abundant SEs signals. This was apparent particularly during the analysis of crude extracts of *N. rustica*. When quite a concentrated sample (1 g of FW plant material per 1 ml) was analysed, only the most prominent SEs were detected, and even then with difficulty. Examination of the MALDI sample support plate revealed a blurred smear instead of a spot, suggesting that the solvent had not fully evaporated before analysis. The remaining solvent evaporates during the laser impact, which must affect the efficiency of sample ionization. As this was not observed during the analysis of a purified sugar ester fraction, this phenomenon was probably caused by the presence of relatively large amounts of hydrophobic cuticular hydrocarbons in the extract. Thus, the concentrations used in the final measurements were reduced to 0.1 g of FW plant material per 1 ml, which clearly improved the results. Further lowering of the sample concentration resulted in poor SEs detection – at a concen-

Table II. Relative composition (given as % of total SEs) and an overview of the possible components of the *N. rustica* sucrose ester fraction. The results are mean values from three analyses \pm SD, based on the peak heights from MALDI-TOF MS experiments.

M_r	$[M + 23]^+$	Relative composition (%)		Prominent acyl groups on glucose ^a		
		Leaves	Fruit capsules	SE-II	SE-III	SE-IV
622	645	–	tr ^b	C ₅ C ₆ C ₆	–	–
636	659	–	1.3 \pm 0.3	C ₆ C ₆ C ₆	C ₄ C ₅ C ₆ C ₅ C ₅ C ₅	–
650	673	5.2 \pm 0.2	4.0 \pm 0.5	–	C ₄ C ₆ C ₆ C ₅ C ₅ C ₆	–
664	687	14.4 \pm 1.1	13.6 \pm 1.0	–	C ₅ C ₆ C ₆	–
678	701	23.2 \pm 2.7	26.6 \pm 1.1	–	C ₆ C ₆ C ₆	–
692	715	15.5 \pm 0.8	14.0 \pm 1.1	–	C ₆ C ₆ C ₇	C ₅ C ₅ C ₆
706	729	9.5 \pm 0.5	6.8 \pm 0.3	–	C ₆ C ₇ C ₇	C ₅ C ₆ C ₆
720	743	9.3 \pm 0.8	8.9 \pm 0.4	–	–	C ₆ C ₆ C ₆
734	757	6.3 \pm 0.7	5.8 \pm 0.3	–	–	C ₆ C ₆ C ₇
748	771	4.9 \pm 0.7	4.3 \pm 0.1	–	–	C ₆ C ₇ C ₇
762	785	6.7 \pm 1.0	7.8 \pm 1.1	–	–	(C ₇ C ₇ C ₇)
776	799	5.0 \pm 1.4	6.9 \pm 0.4	–	–	(C ₇ C ₇ C ₈)

^a On the basis of GC-MS analysis results. The acyl chain patterns given in parentheses were not determined directly, as the compounds did not elute from the GC column.

^b Traces, < 0.4% of the fraction.

tration of 0.01 g FW plant material per 1 ml, SEs signals were only present in the spectra obtained from the *N. rustica* fruit capsule extract. When purified SEs fractions were examined at different concentrations in the range of 0.01–1 g of FW plant material per 1 ml, the relative composition of the fraction remained constant.

Quantitative analysis of SEs in plant material is difficult owing to the lack of commercially available standards of similar structure. Some authors used aliphatic alcohols as internal standards (Severson *et al.*, 1984) or synthesized standards (Einolf and Chan, 1984) prior to GC or CI-MS (chemical ionization-mass spectrometry) analysis. In GC analysis, the use of any internal standard that is distinct from the substances to be determined may lead to inaccurate results, as response factors are hard to estimate. Moreover, if a sample contains SEs that do not elute from the GC column (as in the case of *N. rustica*), the results will cover only part of the fraction. To address these issues, we analysed purified sugar ester fractions from *N. tabacum* and *N. rustica* by MALDI-TOF MS with the addition of sucrose monolaurate as internal standard. However, we did not obtain reliable results, as they varied broadly in terms of both linearity between different sample concentrations and reproducibility between analyses of a single sample. We can only assume that the ionization of sucrose monolaurate was somehow less reproducible than the ionization of more hydrophobic plant SEs. At this stage of experimentation, we cannot state whether or not such an analysis is possible with MALDI-TOF MS. Direct quantitative analysis of SEs using MALDI-TOF MS was unsuccessful, and thus determination of the sensitivity of the method was also impossible. As described above, we determined the SEs content in *N. tabacum* leaves using GC-FID with *n*-docosane as internal standard. Based on these results and on the analysis of extracts containing

known amounts of SEs at concentrations described in the experimental section, we estimated the limit of detection (LOD) of MALDI-TOF MS for the least abundant SEs (M_r 622 and 678) to be as low as ca. 2.0–3.5 $\mu\text{g ml}^{-1}$ of the extract.

Conclusions

The MALDI-TOF MS method for the analysis of plant SEs presented here allows the rapid examination of crude extracts without purification. The analysis of compounds with relatively high molecular weights, not eluting from a standard GC column, is also possible. However, when structural data are required, the analysis should be accompanied by additional GC-MS experiments. Also, the uncertainty of the method in determining the relative composition of the SEs fraction is higher in comparison with a GC-FID analysis. A quantitative analysis using MALDI-TOF MS is, at this stage of experimentation, not possible. The selection of an appropriate internal standard and the validation of the method require additional experiments, which will be carried out in the future.

Acknowledgements

Financial support was provided by the University of Gdańsk under grant 538-8200-0508-1 and by the European Social Fund in the context of the project “Educators for the elite – integrated training program for PhD students, post-docs and professors as academic teachers at University of Gdansk” within the framework of the Human Capital Operational Programme, Action 4.1.1 “Strengthening and development of didactic potential of universities”. This publication reflects the views only of the authors, and the funder cannot be held responsible for any use which may be made of the information contained therein.

Arrendale R. F., Severson R. F., Sisson V. A., Costello C. E., Leary J. A., Himmelsbach D. S., and van Halbeek H. (1990), Characterization of the sucrose ester fraction from *Nicotiana glutinosa*. *J. Agric. Food Chem.* **38**, 75–85.
Chortyk O. T., Pomonis J. G., and Johnson A. W. (1996), Syntheses and characterizations of insecticidal sucrose esters. *J. Agric. Food Chem.* **44**, 1551–1557.

Chortyk O. T., Kays S. J., and Teng Q. (1997), Characterization of insecticidal sugar esters of *Petunia*. *J. Agric. Food Chem.* **45**, 270–275.
Cohen L. H. and Gusev A. I. (2002), Small molecule analysis by MALDI mass spectrometry. *Anal. Bioanal. Chem.* **373**, 571–586.
Ding L., Xie F., Zhao M., Xie J., and Xu G. (2006), Rapid characterization of the sucrose esters from oriental

- tobacco using liquid chromatography/ion trap mass spectrometry. *Rapid Commun. Mass Spectrom.* **20**, 2816–2822.
- Einolf W. N. and Chan W. G. (1984), Estimation of sucrose esters in tobacco by direct chemical ionization mass spectrometry. *J. Agric. Food Chem.* **32**, 785–789.
- Goffreda J. C., Mutschler M. A., and Tingey W. M. (1988), Feeding behavior of potato aphid affected by glandular trichomes of wild tomato. *Entomol. Exp. Appl.* **48**, 101–107.
- Goffreda J. C., Mutschler M. A., Avé D. A., Tingey W. M., and Steffens J. C. (1989), Aphid deterrence by glucose esters in glandular trichome exudate of the wild tomato, *Lycopersicon pennellii*. *J. Chem. Ecol.* **15**, 2135–2147.
- Hare J. D. (2005), Biological activity of acyl glucose esters from *Datura wrightii* glandular trichomes against three native insect herbivores. *J. Chem. Ecol.* **31**, 1475–1491.
- King R. R., Singh R. P., and Boucher A. (1987), Variation in sucrose esters from the type B glandular trichomes of certain wild potato species. *Am. Potato J.* **64**, 529–534.
- King R. R., Calhoun L. A., Singh R. P., and Boucher A. (1990), Sucrose esters associated with glandular trichomes of wild *Lycopersicon* species. *Phytochemistry* **29**, 2115–2118.
- King R. R., Calhoun L. A., Singh R. P., and Boucher A. (1993), Characterization of 2,3,4,3'-tetra-O-acylated sucrose esters associated with the glandular trichomes of *Lycopersicon typicum*. *J. Agric. Food Chem.* **41**, 469–473.
- Li S., Song Z., Liu Z., and Bai S. (2008), Characterization and insecticidal activity of sucrose octanoates. *Agron. Sustain. Dev.* **28**, 239–245.
- Liu T.-X. and Stansly P. A. (1996), Toxicological effects of selected insecticides on *Nephaspis oculatus* (Col., Coccinellidae), a predator of *Bemisia argentifolii* (Hom., Aleyrodidae). *J. Appl. Entomol.* **120**, 369–373.
- Matsuzaki T., Shinozaki Y., Suhara S., Ninomiya M., Shigematsu S., and Koiwai A. (1989), Isolation of glycolipids from the surface lipids of *Nicotiana bigelovii* and their distribution in *Nicotiana* species. *Agric. Biol. Chem.* **53**, 3079–3082.
- Michaud J. P. and McKenzie C. L. (2004), Safety of a novel insecticide, sucrose octanoate, to beneficial insects in Florida citrus. *Florida Entomol.* **87**, 6–9.
- Neal J. J., Tingey W. M., and Steffens J. C. (1990), Sucrose esters of carboxylic acids in glandular trichomes of *Solanum berthaultii* deter settling and probing by green peach aphid. *J. Chem. Ecol.* **16**, 487–497.
- Ohya I., Sinozaki Y., Tobita T., Takahashi H., Matsuzaki T., and Koiwai A. (1994), Sucrose esters from the surface lipids of *Nicotiana cavicola*. *Phytochemistry* **37**, 143–145.
- Peralta I. E., Knapp S., and Spooner D. M. (2006), Nomenclature for wild and cultivated tomatoes. *Rep. Tomato Genet. Coop.* **56**, 6–12.
- Pittarelli G. W., Buta J. G., Neal J. W. Jr., Lusby W. R., and Waters R. M. (1993), Biological pesticide derived from *Nicotiana* plants. US #5,260,281.
- Severson R. F., Arrendale R. F., Chortyk O. T., Johnson A. W., Jackson D. M., Gwynn G. R., Chaplin J. F., and Stephenson M. G. (1984), Quantitation of the major cuticular components from green leaf of different tobacco types. *J. Agric. Food Chem.* **32**, 566–570.
- Severson R. F., Arrendale R. F., Chortyk O. T., Green C. R., Thome F. A., Stewart J. L., and Johnson A. W. (1985), Isolation and characterization of the sucrose esters of the cuticular waxes of green tobacco leaf. *J. Agric. Food Chem.* **33**, 870–875.
- Severson R. F., Chortyk O. T., Stephenson M. G., Akey D. H., Neal J. W. Jr., Pittarelli G. W., Jackson D. M., and Sisson V. A. (1994), Characterization of natural pesticide from *Nicotiana gosssei*. In: *Bioregulators for Crop Protection and Pest Control*, ACS Symposium Series (Hedin P., ed.). American Chemical Society, Washington, USA, pp. 109–121.
- Simmons A. T. and Gurr G. M. (2005), Trichomes of *Lycopersicon* species and their hybrids: effects on pests and natural enemies. *Agric. Forest Entomol.* **8**, 1–11.
- Simonovska B., Srbinoska M., and Vovk I. (2006), Analysis of sucrose esters – insecticides from the surface of tobacco plant leaves. *J. Chromatogr. A* **1127**, 273–277.
- Weinhold A. and Baldwin I. T. (2011), Trichome-derived O-acyl sugars are a first meal for caterpillars that tags them for predation. *PNAS* **108**, 7855–7859.