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Comprehensive chemical derivatization for gas chromatography–mass spectrometry-based multi-targeted profiling of the major phytohormones

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Abstract

In the present investigation we report selection of the *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) reagent as the most comprehensive derivatization protocol among 17 tested reactions covering trifluoroacetylation, pentafluorobenzylation, methylations, and trimethylsilylations. MTBSTFA allowed easy and robust *tert*.-butyldimethylsilyl derivatization of 1-aminocyclopropane-1-carboxylic acid, indole-3-acetic acid, (±)-jasmonic acid, salicylic acid, (±)-abscisic acid, *meta*-topolin, and *trans*-zeatin. Detection limits as analysed by selected ion monitoring quadrupole GC–MS were 0.2, 0.01, 1.0, 0.02, 0.3, 0.3, and 0.9 pmol of injected substance, respectively. Analysis of gibberellic acid A3, *trans*-zeatin riboside and (±)-abscisic acid-β-D-glucopyranosyl ester was best when coupled by splitting extracts and trimethysilylation. The MTBSTFA derivatization protocol was optimised, and validated. The preparation was insensitive to 2% residual water and to ≤1 day storage at room temperature. The final scheme was highly reproducible and successfully applied to extracts from ~300 mg (fresh mass) of tobacco (*Nicotiana tabacum*) root and *Arabidopsis thaliana* seedling.

Keywords: Derivatization, GC; *Nicotiana tabacum*; *Arabidopsis thaliana*; Tobacco; Organic acids; Plant hormones; Auxin; Cytotoxin

1. Introduction

Identification of auxin, the first phytohormone discovered by Went in 1928 [1], spurred a strong and lasting interest in fundamental research on plant growth regulators and applications in biotechnology. In succession abscisic acid, gibberellins, cytokinins, ethylene, jasmonic acids and salicylic acid were described, identified and demonstrated to exhibit respective regulatory functions. Even recently novel signalling substances such as brassinolides and the oligopeptide systemin [2] were found in plants.

Past and recent analysis of phytohormone action led to the emergence of the concept that none of the crucial biological functions, for example growth rate, growth orientation, development, and water balance, could be completely explained in a mono-causal manner. In contrast interplay of phytohormone levels nowadays appears to be more important to our understanding of phytohormone function than absolute concentrations of any single substance [3,4]. This novel insight was the incentive for our effort to

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establish multi-targeted phytohormone profiling as an extension to our recently introduced technology of systems analysis, the GC–MS profiling of primary metabolites [5,6].

Today novel developments in quantitative phytohormone analysis are directed at either multi-parallel analysis or at increased sensitivity without compromising selectivity of detection. Downscaled sample requirement will increase spatial resolution phytohormone analysis. In contrast, multi-parallel analysis will allow novel insights into the interplay of phytohormone action. Our final goal is the efficient, sensitive, and comprehensive multi-targeted quantification of phytohormones from a single sample.

Several publications have already addressed the challenge of developing a suitable method for phytohormone profiling based on instrumental analytical technologies [7–14]. Phytohormones, like most constituents of signal transduction pathways, are trace compounds. Thus phytohormone analysis is subject to the common complications in trace analysis, namely laborious multi-step clean-up procedures, strong influence of sample matrix and ambient conditions [15]. The analytical platform of choice was gas chromatography coupled to mass spectrometry because of unsurpassed instrumental versatility, selectivity, sensitivity, and long-standing previous application in phytohormone analysis. Novel coupling technologies like solid-phase micro extraction, GC–GC coupling, and MS–MS techniques extend the already ample instrumental toolbox towards further means of micro-concentration and micro-separation.

Appropriate and stable derivatization of non-volatile compounds is crucial for successful GC analysis. Indeed nearly all major classes of phytohormones comprise polar compounds with high boiling points. A wide range of derivatizing protocols are available from comprehensive compendium guides [16,17]. Some have already been successfully applied to analysis of different phytohormone classes. Trifluoroacetylation was used in cytokinin analysis [18]. Trimethylsilylation was applied to cytokinin [19] and auxin [20] analysis. *tert*.-Butyldimethylsilylation of cytokinins was reported previously [21]. Alkylation with pentafluorbenzylbromide was successfully applied to the quantification of cytokinin [22] and auxin [23]. Methylation with diazomethane was reported in publications on jasmonic acid [24], auxin, salicylic acid, and abscisic acid [14]. Two-step procedures consisting of alkylation with diazomethane and subsequent trimethylsilylation were described for auxin [25] and gibbberellins [26]. A brief summary of further analytical methods developed for the quantification of the major phytohormones can be found in Ref. [27].

In the present study we reinvestigated and compared those chemical modification schemes which are in frequent use for the GC–MS analysis of phytohormones and which appeared to be versatile. In order of priority, the tested reagents were selected according to ease of handling, comprehensiveness of derivatization, and molar response ratio of the derivatives. The most promising scheme of a multi-parallel analysis was further optimised, validated, and standardised with a representative selection of phytohormones and other chemically related reference substances. Finally, we applied our method to plant matrices using a previously published extraction and clean-up procedure [14]. We introduce a sensitive, robust and easy-to-handle derivatization scheme appropriate for routine analysis of the major phytohormone classes from single plant samples.

2. Experimental

2.1. Standards and reagents

1-Aminocyclopropane-1-carboxylic acid (ACC; CAS 22059-21-8), myo-inositol (INO; CAS87-89-8), (±)-jasmonic acid (JA; CAS 3572-66-5), DL-tryptophan (Trp; CAS 54-12-6), gibberellic acid A3 (GA3; CAS 77-06-5), 5α-cholestane (CH; CAS 481-21-0), *n*-nonadecane (CAS 629-92-5), DL-α-tocopheryl acetate (CAS 7695-91-2) and the pesticide standard mixtures 8081 and EPA 508/508.1 were purchased from Sigma–Aldrich, Munich, Germany; *meta*-topolin (mT) and 24-epibrassinolide (BL; CAS 78821-43-9) were ordered from Duchefa, Haarlem, Netherlands; *trans*-zeatin (Z; CAS 1637-39-4), indole-3-acetic acid (IAA; CAS 87-51-4) and salicylic acid (SA; CAS 69-72-7) were from Merck, Darmstadt, Germany; (±)-abscisic acid (ABA; CAS 14375-45-2), (±)-abscisic acid-β-D-glucopyranosyl

ester (ABA-GE) and *trans*-zeatin riboside (ZR; CAS 6025-53-2) were received from Apex Organics Ltd., Honiton, UK. Where available, chemical abstracts system (CAS) registry numbers of the reference substances are provided.

The reagents were purchased as follows: *N*-methyl-*N*-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), *N*-methyl-*N*-(trimethylsilyl)heptafluorobutyramide (MSHFBA), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), *N*,*N*-dimethylformamidedimethylacetale (DMF-DMA), and trimethylsulphonium hydroxide (TMSH) were from Macherey-Nagel, Düren, Germany. *N*,*O*-Bis-(trimethylsilyl)acetamide (BSA), pentafluorobenzylbromide (PFBBr), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), *N*-trimethylsilyl-imidazole (TSIM), trimethylchlorosilane (TMCS), hexamethyldisilazane (HMDS), *N*-methylbis(trifluoroacetamide) (MBTFA), trimethylphenylammoniumhydroxide (TMAH), and methyl iodide (MeI)–potassium carbonate were ordered from Fluka, Buchs, Switzerland. Pyridine, methanol, dichlormethane (DCM), chloroform, ethylacetate (EtOAc) and formic acid, all HPLC grade, were supplied by J.T. Baker, Philipsburg, NJ, USA. Diazomethane was synthesised as described by Schlenk and Gellerman [28].

2.2. Sample preparation

2.2.1. Derivatization protocols

Standard stock solutions for the comparison of derivatization protocols were prepared in methanol at concentrations of 1 mg/ml. Only Z required addition of 1% (v/v) formic acid. A 5-μl sample of a 1:2 (v/v) dilution of each reference substance was combined with an equal volume of a 1:10 dilution of the 5α-cholestane stock solution, dried under nitrogen, and subjected to the derivatization procedures described below. Final amounts of 0.083 μg of each reference substance were used for GC–MS analysis. In the experiment addressing possible side product formation of MSTFA, BSTFA, MTBSTFA and MSHFBA reactions 0.166 μg SA was used (Table 2). Reaction parameters, e.g. solvent, volume ratios, incubation time and temperature, were not optimised for the initial screening. Instead general manufacturer's recommendations were applied unless indicated otherwise.

2.2.1.1. Trifluoroacetylation with MBTFA

Dissolve in 100 μl EtOAc, add 25 μl reagent, and heat to 120 °C for 2 min before analysis.

2.2.1.2. Methylation with diazomethane [28]

Add saturated ethereal solution of diazomethane, until yellow color is persistent, evaporate sample under nitrogen, and dissolve in 100 μl chloroform for GC–MS.

2.2.1.3. Methylation with DMF-DMA

Dissolve in 100 μl EtOAc, add 1000 μl DMF-DMA in pyridine 1:1 (v/v), and inject for GC–MS analysis when the solution becomes clear after 0.5–3 min.

2.2.1.4. Methylation with TMAH

Dissolve in 100 μl EtOAc, add 1 μl reagent, incubate for 10 min, and analyse directly by GC–MS.

2.2.1.5. Methylation with MeI–potassium carbonate [29]

Dissolve in 40 μl MeI–EtOAc (1:1, v/v), add 1–2 mg potassium carbonate, incubate for 1 h at 90 °C, and inject clear supernatant.

2.2.1.6. Methylation with TMSH

Dissolve in 100 μl EtOAc, add 50 μl reagent, incubate for 10 min at 100 °C and analyse by GC–MS.

2.2.1.7. Silylation with TSIM, MSTFA, BSTFA, MTBSTFA, and MSHFBA [30]

Add 100 μl reagent, incubate for 30 min at 90 °C, and analyse by GC–MS.

2.2.1.8. Silylation with HMDS–TMCS–pyridine (3:1:9, v/v/v) [31]

Dissolve in 100 μl of 3:1:9 (v/v/v) reagent mixture and incubate for 30 min before analysis by GC–MS.

2.2.1.9. Silylation with BSA–TSIM–TMCS, HMDS–TMCS (1:1, v/v) and HMDS–TMCS–pyridine (1:1:1, v/v/v) [31]

Dissolve in 100 μl of 1:1:1 (v/v/v) reagent mixture and incubate 1 h before analysis by GC–MS.

All procedures were carried out in at least three replications and performed at room temperature if not indicated differently.

2.2.2. Optimisation of the MTBSTFA protocol

Analysis of reproducibility, incubation time, incubation temperature, and the search for an internal standard substance with improved performance were carried out with 5 μl of 0.5-mg/ml stock solutions of each reference substance, *n*-nonadecane, and DL-α-tocopheryl acetate which were combined with 3 μl of a 0.1-mg/ml solution of 5α-cholestane. Samples were dried under a stream of nitrogen and incubated in 25 μl MTBSTFA reagent prior to quadrupole GC–electron ionization impact (EI)-MS analysis. Incubation was checked at 40, 60, 80, 100, and 120 °C temperature and at 30, 60, 120, and 180 min reaction time ($n=7$). All subsequent experiments were performed with optimised conditions, namely 1 h at 100 °C. Pesticide standard mixtures 8081 and EPA 508/508.1 were tested for potential candidate standard substances by adding 5 μl of each commercial preparation after heating.

Samples for storage stability tests were prepared as described above in 25 μl MTBSTFA and sealed in GC vials until further analysis. Storage was either at room temperature, 20–25 °C, or at −20 °C. A parallel set was sealed with 0.5 μl water added prior to incubation. Three replications of each set were analysed 5 h, 12 h, 24 h, 3 days, 7 days, and 14 days after start of incubation. For analysis of variance (ANOVA) the 5–24-h measurements were combined into the level ''≤1 day'' of the factor storage time, while the results of days 3–14 comprised the alternate level. Analysis of variance was performed with the statistical software package S-Plus 2000 standard edition release 3 (Insightful, Seattle, WA, USA).

Calibration curves and limits of detection were performed using multiple-component samples which were prepared by dilution of independent stock solutions.

2.2.3. Phytohormone profiling

Tobacco, *Nicotiana tabacum* cv. *Samsun*, plants were grown in sand under optimum growth chamber conditions. Roots were harvested 3 months after germination, rinsed under tap water until free of sand, and were then snap frozen in liquid nitrogen. *Arabidopsis thaliana* seedlings were germinated under sterile conditions on solid support and harvested after 2–3 weeks. For the purpose of this investigation representative batches were sampled, homogenised in a mortar under liquid nitrogen, and stored at −80 °C. Then 300 mg frozen fresh mass of these samples were extracted in 10 ml/g fresh mass of Bieleski solvent pre-cooled to −20 °C [32].

Co-purification of phytohormones from plant extracts was done exactly as described previously [14], except omitting the silica-based aminopropyl purification step. No further attempts at optimisation were undertaken. The final preparation was concentrated by vacuum centrifugation, 1 min at 200 mbar followed by 10 mbar to dryness. The optimised reaction protocol was applied and the MTBSTFA derivatives were analysed by quadrupole GC–EI-MS in the selected ion monitoring (SIM) mode and ion trap GC–chemical ionization (CI)-MS–MS in MS–MS reaction monitoring mode.

2.3. GC–MS analyses

GC–MS systems used in the present work were (i) an MD 800 GC–MS system (ThermoFinnigan, San Jose, CA, USA) with quadrupole technology supplied with split/splitless injection and MassLab software Version 1.4 and (ii) an ion trap Saturn 2000 GC–MS system (Varian, Palo Alto, CA, USA) supplied with programmed temperature vaporization injection and Saturn workstation software version 5.4. AMDIS software was employed to support peak finding before quantitative analysis and for automated deconvolution of reference mass spectra [33]. Identification of derivatives and side products was performed by co-chromatography and mass spectral fragmentation. The identification was supported by comparison to mass spectra presented in Ref. [34] as well as to a commercial mass spectral library in NIST98 format [35]. The quadrupole system was chosen for the analysis of side product formation (Table 2), because mass spectral deconvolution

software in combination with this technology allowed improved automated detection and better mass spectral comparisons with available libraries as compared to ion trap recordings. Information on a data file in the interchange format for AMDIS and NIST98 containing all mass spectra mentioned in Table 2 and respective ion trap mass spectra is to be found in Appendix A as supplementary data for cross-referencing.

Quadrupole GC–MS chromatograms were monitored by electron impact ionisation and either total ion monitoring, m/z 40–600, or in the experiments performed to determine calibration curves and detection limits via segmental selective ion monitoring (GC–EI-SIM-MS). Selected fragments were m/z 272 (ACC), m/z 232 (IAA), m/z 133 (JA), m/z 309 (SA), m/z 190 (ABA), m/z 469 (mT), m/z 302 (Z), and m/z 217 (5α-cholestane).

During initial analyses of derivatization protocols the ion trap mass spectrometer was operated in the EI-MS mode with total ion monitoring, m/z 40–600. Phytohormone profiles of plant samples were monitored in the CI-MS–MS mode with methanol reactant gas and positive ion detection. Maximum reaction time was 128 ms, maximum ionisation time 2 ms, scan rate 0.38 s/scan, multiplier offset 300 V, and emission current 30 μA, and the resonant waveform type was adjusted to MS–MS mode with a parent ion selection window of three atomic mass units. Parent ion selections and excitation amplitudes were segmental and changed as follows: ACC $[M+H]^+$, m/z 330, excitation amplitude 0.6 V; SA $[M+H]^+$, m/z 367, excitation amplitude 0.6 V; JA $[M+H]^+$, m/z 325, excitation amplitude 0.5 V; IAA $[M+H]^+$, m/z 290, excitation amplitude 0.5 V; ABA $[M-H_2O+H]^+$, m/z 361, excitation amplitude 0.6 V.

Arylene type 5% phenyl–95% methylpolysiloxane fused-silica capillary columns were chosen. A 30 m Rtx-5Sil MS fused-silica column, 0.25 mm inner diameter, 0.25 μm film thickness, supplied with a 10 m guard column (Restek, Bad Homburg, Germany) was used for the tests of different reagents and derivatization protocols. The GC–MS system was preconditioned each time the reagents were changed. Optimisation of the MTBSTFA protocol and phytohormone profiles of plant samples were performed without changes in performance on less expensive 30 m DB 5-MS fused-silica columns with 0.25 mm inner diameter, 0.25 μm film thickness (Agilent Technologies, Waldbronn, Germany).

Injection was hot splitless at 230 °C with an oven temperature ramp of 6 °C/min from 70 to 350 °C, ion source temperature was set to 230 °C, and transfer line was at 260 °C. Helium carrier gas was used at a flow-rate of 1 ml/min. These settings were used for all reagents and represent a compromise of previously described analyses [18–26]. The GC method was designed to cover a high temperature range and when tested still separated at least two derivatives from a commercial (±)-jasmonic acid isomer mixture.

The Saturn 2000 System was operated with a temperature program for controlled vaporization after injection, 0.5 min at 110 °C followed by a 250 °C/min ramp to 230 °C.

2.4. Definitions and calculations

Response was defined as chromatographic peak areas derived from mass spectrometric total ion, selected ion, or MS–MS recordings. Molar response was calculated as the quotient of analyte response over mole of substance injected into the GC–MS systems. Molar amount of injected substance was estimated by initial weight, dilution factor before derivatization, final volume of derivative and volume injected. Molar response ratios were the quotients of the molar responses of reference substances and a non-derivatized internal standard substance like 5α-cholestane. The MTBSTFA reaction procedure was optimised (Fig. 1) and tested for robustness by monitoring the relative yield of each derivative. The relative yield was calculated for each phytohormone as percentage of the maximum molar response ratio of the respective main derivative.

3. Results and discussion

3.1. Comparison of derivatization protocols

The reference substances for the following investigations were selected to cover most phytohormone classes by a single commercially available and affordable, naturally occurring compound. Thus the

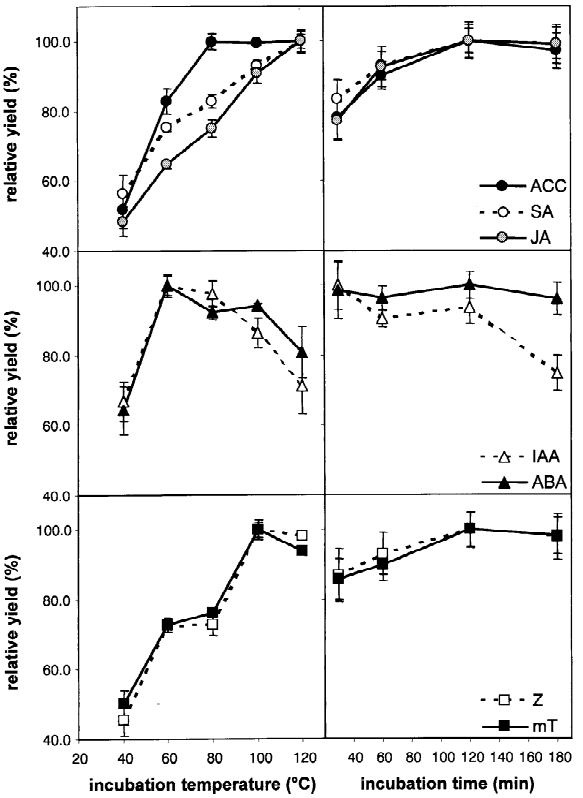

Fig. 1. Optimisation of the MTBSTFA reaction protocol. Relative yield was defined as % of maximum molar response ratio of each main derivative when monitored by quadrupole GC–EI-MS in total ion monitoring mode. Amounts per analysis were 2.5 μg of reference substance and 1.5 μg of 5α-cholestane. Permutation of incubation temperature ($n=7$); permutation of incubation time ($n=7$).

seven major phytohormone classes were represented by IAA, JA, SA, ABA, Z, mT, GA3, and BL (Table 1). Systemin had to be excluded because GC–MS technology is clearly unsuited for the analysis of oligo-peptides. The ethylene precursor, ACC, was included instead of the gaseous phytohormone. Furthermore we attempted to represent common phytohormone conjugates and typical functional groups by the two reference substances, ABA-GE and ZR. Reference substances of the equally important amino acid and inositol conjugates were not commercially available. Therefore, we included Trp and INO in order to assess their respective chemical behavior.

The comparative analyses of derivatization reactions necessitated a common substance for internal volume standardization. This substance was required to be inert with respect to all tested reagents. Therefore, initial experiments focused on the use of a range of hydrocarbons (data not shown). 5α-Cholestane was the best choice available with respect to inertness, intermediate volatility and distinctive fragmentation.

In Table 1 we summarise the results of the initial screening of reagents and protocols, among those listed in Section 2.2.1. In cases of multiple derivatives only those with highest molar response ratio are shown. IAA, JA, and ABA were easily detected by all protocols, but no strategy of chemical modification allowed analysis of all reference substances. Best coverage and molar response ratios were obtained with silylating reactions. Combined silylation and methylation increased the number of side products but allowed detection of BL. However, cytokinins, ABA-GE, and ZR were lost. This observation was also made for all stand-alone methylation reactions. Trifluoroacetylation exhibited strong selectivity and low molar response ratios. PFBBr was communicated as a highly sensitive reagent and robust to residual water [36]. In addition, pentafluorobenzylation is ideally suited for negative chemical ionisation (NCI)-MS [16,22]. In our hands PFBBr-derivatives exhibited high sensitivity even when monitored with EI-MS, but allowed detection only of ACC, IAA, JA, SA, ABA, and Trp.

Four silylating reagents with high donor strength, BSTFA, MSTFA, MSHFBA, and MTBSTFA, the later transferring *tert.*-butyldimethylsilyl groups, appeared to be most comprehensive. Comparison with less reactive silylating reagents under mild reaction conditions demonstrated that high reactivity was essential for this observation. Therefore, only those highly reactive reagents were further investigated with regard to the formation of side products and compared in a single large-scale experiment using a quadrupole GC–EI-MS system (Table 2). Most molar response ratios were increased as compared to previous ion trap results (Table 1). This effect was caused to a large extend by a reduced molar response of 5α-cholestane. Interestingly the quadrupole GC–MS system appeared also to discriminate the INO derivative and to be more sensitive to the derivatives of mT, Z, Trp, and ACC. The overall relative standard deviation (RSD) of these experiments was

Table 1
Molar response ratios of the main derivative obtained from 0.083 μg of each reference substance

Reagent[c]	Reference substance[a]												
	ACC	IAA	JA	SA	ABA	ABA-GE	mT	Z	ZR	GA3	BL	Trp	INO
A													
MTBSTFA	**0.146**	**0.237**	**0.149**	**0.270**	**0.262**		**1.042**	**0.440**	0.034	**0.169**		**0.376**	
B													
30–60 Min, room temperature:													
BSA–TMCS–TSIM (1:1:1, v/v/v)[b]		0.005											
HMDS–TMCS–pyridine (1:1:1, v/v/v)[b]		0.090	0.098	0.096	0.009					0.074			
HMDS–TMCS–pyridine (3:1:9, v/v/)[b]		0.073	**0.117**		0.007			0.021		**0.154**			
HMDS–TMCS (1:1, v/v)[b]	0.048	0.069	**0.115**	0.015	**0.121**			0.057		**0.588**			
30 Min, 90 °C:													
TSIM		0.005											
BSTFA	<0.001	**0.179**	**0.157**	**0.153**	**0.197**	0.010		0.044		**0.907**		0.009	**0.596**
MSTFA	0.069	**0.252**	0.096	0.098	**0.175**	0.020	0.014	**0.131**	**0.311**	**0.801**		**0.123**	**0.404**
MSHFBA	0.087	**0.231**	**0.133**	**0.149**	**0.146**	0.029	0.040	0.095	**0.343**	**0.691**		0.088	**0.231**
C													
MeI		0.066	0.026	<0.001						**0.138**			
Diazomethane		0.055	**0.133**	0.007	**0.146**					0.083	0.012		
DMF-DMA		0.050	0.088		**0.130**			0.002		0.088			
TMSH		**0.360**	0.056	0.001	**0.149**			0.066		0.009			
TMAH		**0.116**	**0.105**	0.002	**0.147**			0.009		0.072			
D													
Diazomethane–MSTFA	0.015	**0.179**	0.083	0.047	**0.139**			0.008		**0.643**	0.020		**0.184**
E													
MBTFA		0.087	0.051		0.004					0.012			
F													
PFBBr	**0.288**	0.086	**0.115**	**0.259**	**0.317**							**0.200**	

Molar response ratios were calculated from ion trap EI-MS total ion currents by normalisation to the signal of an equal amount of 5α-cholestane within each preparation. The table was compiled from multiple experiments. Each experiment was performed with aliquots of the same reference substance mixture ($n=3$). Values >0.1 are in bold format. (A) *tert.*-Butyldimethylsilylation; (B) trimethylsilylations; (C) methylations; (D) combined trimethylsilylation and methylation; (E) trifluoroacetylation; (F) pentafluorobenzylation.

[a] ABA, (±)-Abscisic acid; ABA-GE, (±)-abscisic acid-β-D-glucopyranosyl ester; ACC, 1-aminocyclopropane-1-carboxylic acid; BL, 24-epibrassinolide; GA3, gibberellic acid A3; IAA, indole-3-acetic acid; INO, myo-inositol; JA, (±)-jasmonic acid; mT, *meta*-topolin; SA, salicylic acid; Trp, DL-tryptophan; Z, *trans*-zeatin; ZR, *trans*-zeatin riboside.

[b] Volume ratios.

[c] Refer to Section 2.1 for full identification of reagents.

Table 2
Molar response ratios of all observed derivatives generated from 0.083 μg of each reference substance

Reference substance[a]			TBS derivative			TMS derivative				
Compound	Molecular mass (g/mol)	Amount[c] (nmol)	Compound	No.	MTBSTFA	Compound	No.	BSTFA	MSTFA	MSHFBA
ACC	101.1	0.82	ACC TBS 1	2	0.688	ACC TMS 1	2	0.421	0.380	0.460
IAA	175.2	0.47	IAA TBS 1	2	0.005	IAA TMS 1	2	1.152	1.060	1.058
			IAA TBS 2	1	1.369	IAA TMS 2	1	0.140	0.002	0.001
JA	210.3	0.39	JA TBS 1	1	0.675	JA TMS 1	1	0.436	0.293	0.408
			JA TBS 2	2	0.002	JA TMS 2	2	0.002	0.163	0.010
SA	138.1	1.20[b]	SA TBS 1	2	1.464	SA TMS 1	2	0.613	0.473	0.699
ABA	264.3	0.31	ABA TBS 1	1	1.229	ABA TMS 1	1	0.834	0.746	0.693
			ABA TBS 2	2	0.050	ABA TMS 2	2	0.001	0.087	0.018
ABA-GE	426.5	0.19		–	–	ABA-GE TMS 1	(4)	0.023	0.008	0.015
mT	241.4	0.34	mT TBS 1	2	1.421	mT TMS 1	2	0.595	0.620	0.619
				–	–	mT TMS 2	1	0.030	0.039	0.025
				–	–	mT TMS 3	3	0.005	0.017	0.018
Z	219.2	0.38	Z TBS 1	2	1.856	Z TMS 1	2	1.058	1.057	0.966
			Z TBS 2	3	0.007	Z TMS 2	3	0.050	0.079	0.077
ZR	351.4	0.24	ZR TBS 1	(3)	0.016	ZR TMS 1	4	1.548	1.494	1.561
GA3	346.4	0.24	GA3 TBS 1	1	0.100	GA3 TMS 1	3	0.999	1.066	0.950
				–	–	GA3 TMS 2	3	0.081	0.077	0.057
BL	480.8	0.17		–	–		–	–	–	–
Trp	204.2	0.41	Trp TBS 1	3	0.005	Trp TMS 1	3	0.329	1.175	1.270
			Trp TBS 2	2	1.254	Trp TMS 2	2	0.053	0.191	0.083
				–	–	Trp TMS 3	1	0.030	<0.001	<0.001
				–	–	Trp TMS 4	2	0.160	<0.001	<0.001
INO	180.2	0.46		–	–	INO TMS 1	6	0.009	0.137	0.068

Molar response ratios were calculated from quadrupole EI-MS total ion currents of a single experiment ($n=3$) by normalisation to the signal of an equal amount of 5α-cholestane within each preparation. Mass spectra of all *tert*.-butyldimethylsilyl (TBS) and trimethylsilyl (TMS) derivatives mentioned in the table are available on request from the communicating author from the mass spectral library included as supplementary data in Appendix A. The number of trimethylsilyl groups (No.) is listed, brackets indicate an estimated number.

[a] ABA, (±)-Abscisic acid; ABA-GE, (±)-abscisic acid-β-D-glucopyranosyl ester; ACC, 1-aminocyclopropane-1-carboxylic acid; BL, 24-epibrassinolide; GA3, gibberellic acid A3; IAA, indole-3-acetic acid; INO, myo-inositol; JA, (±)-jasmonic acid; mT, *meta*-topolin; SA, salicylic acid; Trp, DL-tryptophan; Z, *trans*-zeatin; ZR, *trans*-zeatin riboside.

[b] 0.166 μg per preparation.

[c] Amount per analysis.

21, 31, 27, and 24% ($n=3$) including all minor products of the reactions with, MTBSTFA, BSTFA, MSTFA, and MSHFBA, respectively. The most comprehensive derivatization was trimethylsilylation. All trimethylsilyl reagents, BSTFA, MSTFA, and MSHFBA, generated a single main product and identical side products except for ABA-GE and BL. In the case of ABA-GE low signal intensity was likely caused by instability of the conjugate as judged by occurrence of free silylated glucose (data not shown). In comparison to trimethylsilyl reagents, MTBSTFA was slightly more sensitive and less prone to formation of side products (Table 2; refer to Trp and mT). In some cases MTBSTFA exhibited a preference for a lower degree of substitution, namely IAA and Trp. Unfortunately this property of

MTBSTFA did not allow analysis of GA3, ZR, ABA-GE, BL, or INO. In the case of GA3 and ZR we detected minor signals of derivatives with low degree of substitution, but the bulk derivative was lost.

In view of the ultimate goal of our efforts—the sensitive close to comprehensive multi-targeted quantification of phytohormones—we decided on an in-depth analysis of the MTBSTFA derivatization reaction. This decision took into account first the prospective sensitivity, namely the combined aspects of low side product formation, high molar response and low complexity of fragmentation. Secondly we expected higher selectivity of detection, because the mass spectral fragmentation pattern of MTBSTFA derivatives generates typical $[M-57]^+$ and $[M-15]^+$ fragments from, in most cases, still detectable molecular ions. Finally we took into account the purity of the reagent and the stability of derivatives [37]. The drawback of MTBSTFA derivatization, however, is low sensitivity for gibberellins and monosaccharide conjugates. This deficiency may be circumvented by splitting of extracts and in parallel alternative analysis by trimethylsilylation. Trimethylsilylation has a more comprehensive potential [5], but showed interference with residual water. Moreover, mass spectral fragmentation patterns were clearly more complex and less specific. Therefore a higher demand on pre-purification and concentration from plant matrices and thus lower overall sensitivity was expected.

3.2. Optimisation of the MTBSTFA protocol

3.2.1. Repeatability of GC–MS analysis

The MTBSTFA protocol was optimised using a quadrupole GC–EI-MS system because of the higher sensitivity in the EI-MS mode and comparative ease of handling and data processing. For this purpose we performed experiments of nine repeated injections in the course of 10 h with a reference mixture of ACC, IAA, JA, SA, ABA, mT, and Z and varying internal standard substances. The molar response ratios of the derivatives using our initial choice of the 5α-cholestane standard had 6.0–13.1% RSD (Table 3). In our hands this level of repeatability is typically achieved by GC–EI-MS systems, when MTBSTFA or MSTFA are used as solvents for injection.

In an attempt to assess possible improvement of GC–MS reproducibility we tested internal standardization by *n*-nonadecane, DL-α-tocopheryl acetate, and each of the components of the pesticide standard mixtures 8081 and EPA 508/508.1. The pesticide mixtures allowed the fast screening of a large range of different compound classes. which were in part derivatized by MTBSTFA. None of the tested compounds exhibiting higher as well as lower boiling points qualified for a better internal standard

Table 3
Repeatability of the molar response ratio of the main derivative synthesised from 0.083 μg of each reference substance

Reference substance[a]			Total ion response, molar response ratio	
Compound	Amount[b] (nmol)	Derivative	Average	RSD (%)
ACC	0.82	ACC TBS 1	1.004	6.0
IAA	0.47	IAA TBS 2	1.281	13.1
JA	0.39	JA TBS 1	0.857	6.2
SA	0.60	SA TBS 1	0.920	6.8
ABA	0.31	ABA TBS 1	1.438	8.7
mT	0.34	mT TBS 1	1.621	11.8
Z	0.38	Z TBS 1	2.040	13.0
Trp	0.41	Trp TBS 2	1.300	10.7
CH	0.48	–	–	–

Molar response ratios were calculated from quadrupole EI-MS total ion currents ($n=9$) set to a scanning range of m/z 40–600 using the signal of 5α-cholestane within each preparation for normalisation.

[a] ABA, (±)-Abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; CH, 5α-cholestane; IAA, indole-3-acetic acid; JA, (±)-jasmonic acid; mT, *meta*-topolin; SA, salicylic acid; Trp, DL-tryptophan; Z, *trans*-zeatin.

[b] Amount per analysis.

of any of the reference derivatives than 5α-cholestane. Therefore, we continued internal standardization with this compound.

3.2.2. Optimisation of reaction conditions

Fig. 1 summarises the effects of permuted incubation time and temperature on relative yield. The relative yield was calculated separately for the main derivatives of each phytohormone. The maximum molar response ratio obtained in each experiment was set to 100% relative yield. Side products did not accumulate under any of the conditions tested. Derivatives were grouped according to similarity of behavior. These groups were related to the GC elution sequence of derivatives. Cytokinins exhibited almost identical behavior, and IAA and ABA were highly similar, whereas JA, SA, and ACC showed a similar tendency. In general incubation temperature exhibited a stronger influence on reaction yield than incubation time. The optimum compromise for all phytohormones was 1-h incubation at 100 °C.

3.2.3. Stability and storage

An experiment in factorial 2^3 design was performed to detect parameters which might influence robustness of analysis after final derivatization. Two levels of three factors were investigated by 24 experiments comprising three replications of each possible combination of factors. The stability of the derivative was tested for a typical time of analysis, ≤1 day, as compared to storage for 3–14 days. Influence of residual water, a common problem in phytohormone preparations, was checked by addition of 2% (v/v) of water before reaction with MTBSTFA. Storage temperature was the third parameter tested. Typically samples are exposed to room temperature before GC analysis. Therefore, we compared storage at −20 °C with exposure to room temperature.

A three-way ANOVA was performed for each phytohormone. IAA, SA, and ABA analysis was not influenced by any of the tested challenges to robustness. In the case of IAA and ABA this observation was contrary to our expectations (Fig. 1). Each derivative was stable in the presence of trace amounts of water. All relative yields were 90–100%. Storage time increased relative yield of ACC by a factor of 1.5 ($P<0.001$) and relative yield of JA by a factor of 2.0 ($P=0.004$). In contrast, relative yield of mT was reduced by a factor of 0.65 ($P<0.001$). This finding may be indicative of incomplete derivatization in the case of ACC and JA and shows slight long-term instability of the mT derivative. Lowering storage temperature to −20 °C appeared not to be beneficial. In contrast the relative yield of Z was reduced by a factor of 0.9 ($P=0.006$). This effect was increased in combination with long storage time ($P<0.001$). No other interaction of factors was

Table 4
Detection limits of phytohormones expressed as amount required before derivatization by MTBSTFA[a]

Phytohormone	Selected ion monitoring					
	Derivative	Fragment (m/z)	Relative abundance (%)[c]	Detection limit[b] (ng)	Detection limit[b] (pmol)	S/N
ACC	ACC TBS 1	272	7.3	0.50	5.0	4:1
IAA	IAA TBS 2	232	19.4	0.05	0.3	5:1
JA	JA TBS 1	133	7.0	5.00	24.0	5:1
SA	SA TBS 1	309	18.5	0.05	0.4	15:1
ABA	ABA TBS 1	190	6.0	2.00	7.5	4:1
mT	mT TBS 1	469	8.9	2.00	8.3	5:1
Z	Z TBS 1	302	7.9	5.00	23.0	4:1

Quadrupole GC–EI-MS was set to selected ion monitoring. The signal-to-noise ratio at the limit of detection was calculated based on the maximum amplitude of the background signal in the vicinity of the respective peak.

[a] ABA, (±)-Abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; IAA, indole-3-acetic acid; JA, (±)-jasmonic acid; mT, *meta*-topolin; SA, salicylic acid; Z, *trans*-zeatin.

[b] Total derivatization volume was 25 μl. A 1-μl sample was applied to GC–MS analysis.

[c] The relative abundance of selected ion fragments was determined in parallel by total ion monitoring experiments with m/z 40–600.

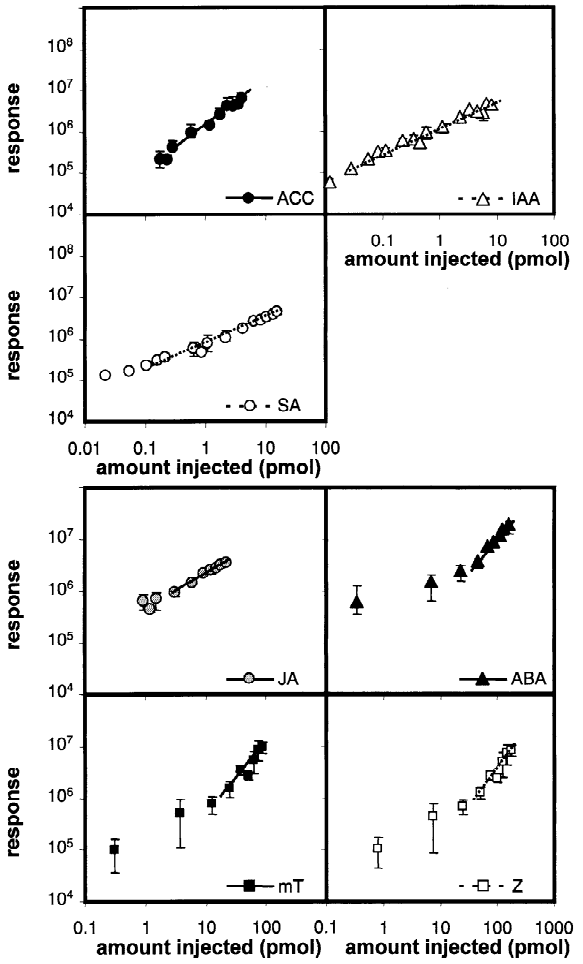

Fig. 2. Calibration curves ($n=6$) demonstrating the working range of the MTBSTFA derivatization protocol of 1-h incubation at 100 °C in a volume of 25 μl, as determined by quadrupole GC–EI-MS set to selected ion monitoring mode. The smallest amounts shown represent the detection limits. Fragments and the signal-to-noise ratios at the detection limits were as listed in Table 4. Error bars represent standard deviation.

detectable. ANOVA clearly demonstrated general robustness of the selected protocol. Furthermore, we checked effects on side product formation and found their relative occurrence to be invariant.

3.2.4. Calibration and limits of detection

Quadrupole GC–EI-MS set to selected ion monitoring mode was used for analysis of detection limits and respective signal-to-noise ratios (S/N). Only 1/25 of final the sample volume was analyzed by GC–MS operated with splitless injection. The noise value was determined as the maximum amplitude of the background signal in a range of ±5 times the respective peak width. Peak height was calculated from the average noise level to peak apex. The detection limits are presented as amount required before derivatization with MTBSTFA reagent (Table 4). Sensitivity varied within two orders of magnitude among the different compounds analysed. Detection of IAA and SA was highly sensitive, 0.3 and 0.4 pmol, respectively, whereas JA and Z exhibited detection limits of 24 and 23 pmol per sample. ACC, ABA, and mT had intermediate detection limits of 5.0, 7.5, and 8.3 pmol. The fragments chosen for selected ion monitoring had relative abundances of 7–19% and were mostly in the high-molecular mass range. Both high relative abundance and high mass of available fragments contributed to the considerable sensitivity of *tert.*-butyldimethylsilyl derivatives as compared to TMS derivatives.

Calibration curves of the phytohormones were determined and are presented in double logarithmic scale (Fig. 2). The smallest amount shown corresponds to the respective detection limits (Table 4). In the sub pmol to pmol range the reference substances, ACC, IAA, SA, and JA, all exhibited clear linear behavior. The response functions of ABA, mT, and Z were sigmoidal. Calibration curves which were extended into the nmol range all had a sigmoidal shape and indicated upper detection limits of 10–100 nmol injected.

3.3. Phytohormone profiling

The MTBSTFA protocol was successfully applied to analysis of extracts from tobacco root and seedlings of *A. thaliana*. A published extraction and purification method [14] was adopted and phytohormone fractions prepared accordingly from representative samples of ~0.3 g fresh mass. Analysis with quadrupole GC–EI-MS in selective ion monitoring mode exhibited in part intense peaks which were identified by spiking experiments. Moreover, each phytohormone was monitored using four different fragments in four consecutive runs. These experiments and total ion scanning analysis indicated inadequate sample purity for routine analysis with quadrupole GC–EI-MS. For the unequivocal demon-

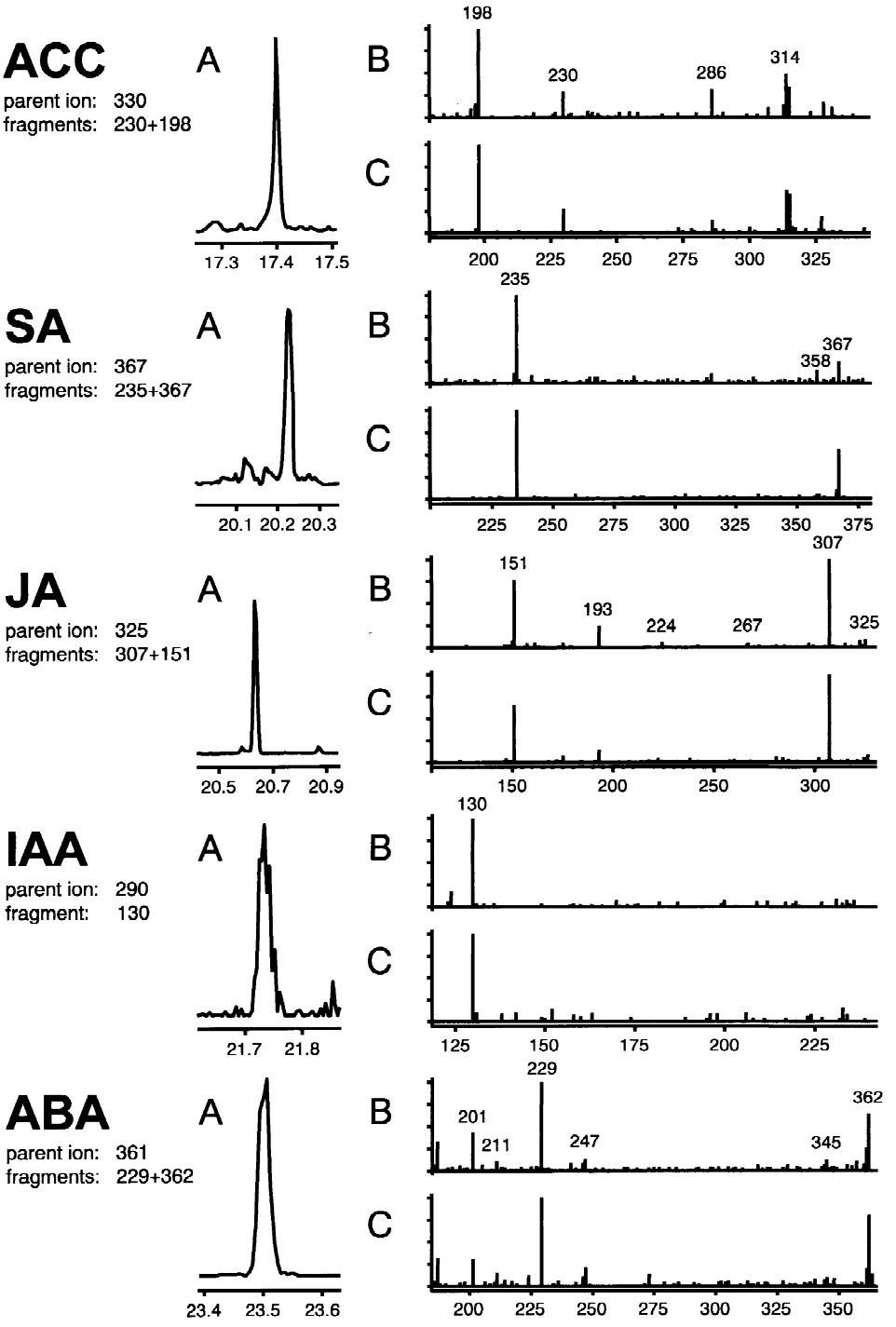

Fig. 3. MTBSTFA phytohormone profile of 0.3 g tobacco (*Nicotiana tabacum*) root, recorded with an ion trap GC system in CI-MS–MS mode. (A) Specific MS–MS fragment traces. MS–MS fragmentation of reference substances (B) are compared to the MS–MS spectra of endogenous plant compounds (C). MS–MS spectra were taken from the peak apexes. ABA, (±)-abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; IAA, indole-3-acetic acid; JA, (±)-jasmonic acid; SA, salicylic acid.

stration of the presence of endogenous phytohormones we employed ion trap GC–CI-MS–MS. Analysis of MS–MS spectra allowed identification of ACC, IAA, SA, JA, and ABA from both tobacco root (Fig. 3) and *A. thaliana* seedlings (data not shown). In shoot organs of *A. thaliana* IAA, SA, and JA, and ABA are typically found in concentrations of 100–1000 pmol/g fresh mass, whereas SA and ABA mostly range from 10 to 100 pmol/g fresh mass [14]. Our successful identification of IAA, SA, JA, and ABA was therefore in agreement with expectations. ACC was previously not noticed in similar preparations (Fig. 3). We did not try to monitor Z in this experiment because the Z concentration reported to occur in tobacco seedlings did not exceed 0.25 pmol/g fresh weight [38].

4. Conclusions

We present a novel method appropriate for comprehensive chemical derivatization and subsequent gas chromatography–mass spectrometry of phytohormones. The coverage of phytohormone classes is broader than reported previously for a single analysis [14]. However, current means of joined extraction and preparation of phytohormone fractions from plant samples restrict the potential of our analysis to five endogenous target compounds, e.g. ACC, IAA, SA, JA, and ABA. Our future efforts will focus on extending the preparation protocol in order to fully exploit our novel method. Currently ion trap GC–CI-MS–MS analysis is mandatory and quantitative standardisation is restricted to the use of stable isotope labelled reference substances.

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Appendix A. Supplementary file 1

The data file, phytoh.msp[1], contains mass spectra of all phytohormone derivatives mentioned in Tables 2–4. Quadrupole and ion trap electron impact ionisation mass spectra are included.

The spectrum name was designed to allow sorting according to the reference substance. For example, the name ABA TBS1≠EI≠Q≠MTBSTFA[2] codes for the name of the reference substance and type of derivative, mode of ionisation, mode of mass spectral detection and reagent. The spectrum ID allows sorting according to reagent, mode of ionisation, mode of mass spectral detection, and source chromatogram, for example MTBSTFA≠EI≠Q≠1235DW21.

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[1] The file format *.msp may be imported into either NIST98 or AMDIS software (to be downloaded from http://chemdata.nist.gov/mass-spc/Srch_v1.7/index.html and http://chemdata.nist.gov/mass-spc/amdis/, respectively). The file phytoh.msp is available on request from the communicating author.

[2] EI, electron impact; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; MTBSTFA, *N*-methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoroacetamide reagent; Q, quadrupole technology; T, ion trap technology.

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