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Analysis of ascorbate and dehydroascorbate in plant extracts by high-resolution selected ion monitoring gas chromatography–mass spectrometry

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Abstract

Here we report on the quantitative analysis of *tert.*-butyldimethylsilyl derivatives, of ascorbate and dehydroascorbate in plant extracts by high-resolution selected ion monitoring gas chromatography–mass spectrometry. The analysis involved an isotope dilution assay using [$^{13}C_1$]ascorbate and [$^{13}C_1$]dehydroascorbate. The limit of detection was approximately 0.5 pg for ascorbate and 5 pg for dehydroascorbate. The reproducibility of the whole quantification procedure, from extraction of *Pinus sylvestris* needles with 5% trichloroacetic acid to the final analysis, was very high: giving a relative standard deviation (R.S.D.) of 4% and 10% for ascorbate and dehydroascorbate, respectively. The reproducibility of the analysis of the redox ratio of ascorbate/(ascorbate+dehydroascorbate) was even higher (R.S.D.=1%). The high-resolution selected ion monitoring gas chromatography–mass spectrometry methodology developed here with very high sensitivity and specificity for ascorbate and dehydroascorbate opens new perspectives for investigations on distribution patterns of these substances in plants.

Keywords: *Pinus sylvestris*; Ascorbate; Dehydroascorbate; Vitamins

1. Introduction

The pivotal role of ascorbate and dehydroascorbate in several physiological processes in plants and mammalian cells has been thoroughly reviewed [1–6]. The role of ascorbate as an oxygen scavenger in the aqueous phase of cells and as a cofactor in structural protein organization is well known and the redox ratio of ascorbate in different tissues has been shown to be an indicator of level and type oxidative stress in plants. For example, Luwe and Heber [7] and Sgherri et al. [8] found that ozone and drought changed the redox state of ascorbate in the apoplast and leaves, respectively. Ascorbate, and enzymes that metabolize ascorbate-related compounds are involved in the control of several plant growth processes as the biosynthesis of hydroxyproline-rich proteins required for the progression of G1 and G2 phases of the cell cycle, the cross-linking of cell wall glycoproteins and other polymers and redox reactions at the plasma membrane [4]. Further, the redox couple of ascorbate/dehydroascorbate may play an essential role the gene regulation. Many regulators in, for example, bacteria and mammalian

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cells, are directly sensitive to oxidation and reduction in vitro including oxyR, soxR, thioredoxin, NF-*k*B and Ap-1 [9]. Dehydroascorbate has also been proposed to play an essential role in the oxidation of thiols in protein in the endoplasmatic reticulum [10].

Several problems arise in the analysis of ascorbate and dehydroascorbate in plant extracts. The compounds have been shown to be degraded, to bind to proteins, and also to be inter-converted by oxidation/reduction during extraction and analysis [11–14]. High-performance liquid chromatographic (HPLC) procedures have been developed for ascorbate quantification but, the need for corrections for losses during purification, low sensitivity and low specificity might all be problems with these methods [14]. Furthermore, most analyses of dehydroascorbate are based on indirect measurements and are performed after its conversion to ascorbate [14–19]. There are also colorimetric/spectrophotometric and fluorometric assays for direct analysis of dehydroascorbate but these methods have low sensitivity and specificity [14].

There is therefore a need for more accurate and sensitive methods of analysis of both ascorbate and dehydroascorbate in plant extracts. Here we present a combined gas chromatography–mass spectrometric (GC–MS) method based on techniques developed for plasma analysis [20]. The quantification involves an isotope dilution technique, based on the response ratio between selected masses for the native compound and the added heavy isotopes of [$^{13}C_1$]ascorbate and [$^{13}C_1$]dehydroascorbate. With this methodology it is possible to analyze both ascorbate and dehydroascorbate in the same sample with very high sensitivity and accuracy.

2. Experimental

2.1. Plant material

Current year *Pinus sylvestris* L. needles were collected in the middle of the day from naturally grown seedlings (4-years old) protruding above the snow outdoors in April. Additionally, current year needles were collected from actively growing 4-year old seedlings in a green house with a photoperiod of 18 h, a day/night temperature of 20°C/15°C and a photon flux density of approximately 200 μmol m^{-2} s^{-1} (HQITS 400, Osram, Germany).

2.2. Standards

Standards of ascorbate and dehydroascorbate were obtained from Sigma. [1-$^{13}C_1$]Ascorbate (99% isotopic purity, 98% chemical purity) was purchased from Larodan and the [1-$^{13}C_1$]dehydroascorbate was obtained from [1-$^{13}C_1$]ascorbate by enzymatically catalyzed reaction with ascorbate oxidase (Sigma) at room temperature overnight (pH 5.6, 0.1 *M* $KHPO_4$, 1 m*M* EDTA). Stock solutions of [1-$^{13}C_1$] of ascorbate and dehydroascorbate (1 mg/ml) were kept at −80°C and used within one week. The stock solutions were routinely checked for impurities and degradation. Milli-Q plus 185 water was used in all experiments (Millipore, France).

2.3. Extraction

Needles for ascorbate measurements were frozen in liquid nitrogen until the analysis was performed. The needles were then transferred to liquid nitrogen in cold steel vials and were ground for 90 s in a dismembrator (Retsch, Germany). The needle powder, still frozen, was transferred to Eppendorf tubes and weighed (approximately 0.1 g FW). The samples were then immediately mixed with 1 ml extraction medium (5% trichloric acid w/v) containing [1-$^{13}C_1$]ascorbate and [1-$^{13}C_1$]dehydroascorbate as internal standards. The internal standards were added to the extraction medium immediately before the extraction, in amounts adjusted to be approximately equal to the non-labeled contents of the samples. The extract was then sonicated for 15 s (Soniprep 150, MSE, UK) and shaken for 15 min at 5°C. After centrifugation (15 000 *g* for 10 min) 0.75 ml was transferred to glass vials and was shaken with 1.5 ml diethyl ether for 1 min. The aqueous phase of the extracts (usually 0.2 ml) were reduced to dryness in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, USA). The dried samples were redissolved in 20 μl pyridine by sonication in an ultra-sound water bath for 5 min. A 20-μl volume of

acetonitrile was added to the samples, which were then incubated with 20 μl N-*tert.*-butyldimethylsilyl-N-methyltrifluoroacetamide+1% *tert.*-butyldimethylchlorosilane (MTBSTFA+1% TBDMCS, Pierce) at 90°C for 1.50–2 h to form *tert.*-butyldimethylsilyl (TBDMS) derivatives. After derivatization the samples were kept at room temperature for 24 h until analyzed by GC–MS.

2.4. Gas chromatography–mass spectrometry

Samples were injected by a Hewlett-Packard 7673 autosampler splitless into a Hewlett-Packard 5890 GC equipped with a 15 m×0.25 mm I.D. fused-silica capillary column with a chemically bonded 0.25 μm CP-SIL-8-MS stationary phase (Chrompack, Middelburg, Netherlands). The injector temperature was 250°C. For the analysis of ascorbate the column temperature was held at 70°C for 2 min, then increased by 30°C min^{-1} to 220°C, and by 3°C min^{-1} to 245°C. For the analysis of dehydroascorbate the column temperature was held at 70°C for 2 min, then increased by 15°C min^{-1} to 200°C, and by 3°C min^{-1} to 215°C. The column effluent was introduced into the ion source of a JEOL JMS-SX102 double-focusing magnetic sector mass spectrometer (Jeol, Tokyo, Japan). The interface and the ion source temperatures were 270°C and 230°C, respectively. Ions were generated with 70 eV at an ionization current of 300 μA. Full-scan mass spectra were obtained at a rate of 1 s per scan for a mass range of 40–800 u. The acceleration voltage was 10 kV.

High-resolution selected ion monitoring (HR-SIM) measurements were performed using accelerating voltage switching from 10 kV. Perfluorokerosene was used as reference compound, choosing a suitable lock mass. The dwell time was 50 ms, and for each compound two ions and two ^{13}C-labeled analogues were recorded; ascorbate, *m/z* 443.2105, 444.2105, 575.3076, 576.3076; dehydroascorbate, 345.1189, 346.1189, 387.1659, 388.1659. The resolution was 10 000.

Calibration curves were recorded from 0.625 ng to 9.375 ng ascorbate/dehydroascorbate with a fixed amount (1.875 ng) of [$^{13}C_1$]ascorbate/dehydroascorbate as the internal standard. All data were processed by a JEOL MS-MP7010D data system.

2.5. Precision

Precision in the GC–MS analyses was estimated by measurement of 0.625 ng to 9.375 ng ascorbate/dehydroascorbate with a fixed amount of [$^{13}C_1$]ascorbate/dehydroascorbate as the internal standard. The integrated area ratio between *m/z* ascorbate/dehydroascorbate and *m/z* [$^{13}C_1$]ascorbate/dehydroascorbate, and the relative standard deviation (R.S.D.) of five replicates were calculated. The reproducibility of the GC–MS analyses of plant extracts was assessed by subsequently analyzing the same extract three times.

The overall precision of ascorbate/dehydroascorbate analysis of plant extracts was estimated by dividing Scots pine needle samples (homogenized in liquid nitrogen) into eleven aliquots, which were then extracted, purified and analyzed by HR-SIM as described in Section 2.4. The mean amount of endogenous ascorbate/dehydroascorbate in each extract and R.S.D. were calculated.

3. Results

Derivatization of ascorbate with MTBSTFA resulted in good recovery, with one major peak of high intensity, shown to be ascorbate with four TBDMS groups, and two minor peaks (probably a result of rearrangement during derivatization). The intensity of the minor peaks was less then 5% of that of the major peak, and they are not therefore a problem in the quantitative analysis of ascorbate. GC–MS analysis of the TBDMS-derivative of dehydroascorbate showed three major peaks if analyzed directly after the derivatization at 90°C. This problem could be overcome by incubating the derivatized samples a further 24 h at room temperature, after which there was only one major peak. This is probably due to a fast formation of $(TMS)_4$- or $(TMS)_5$-dehydroascorbate besides the formation of $(TMS)_2$-dehydroascorbate. However, the equilibrium is toward the $(TMS)_2$-dehydroascorbate, resulting in fewer peaks after 24 h. It should be emphasized that the total ion current of the TBDMS-dehydroascorbate was approximately 10% of that of the TBDMS-ascorbate, resulting in less sensitive analysis of dehydroascorbate.

A problem in the analysis of dehydroascorbate was due to impurity of the commercially available standards. This has also been reported by Deutsch and Kolhouse [20]. Three different vials bought on three different occasions were tested by comparing them, assessed on dry material, with ascorbate oxidized to dehydroascorbate by ascorbate oxidase. Only one was sufficiently pure to be useful as a standard in the quantification of dehydroascorbate.

Electron impact mass spectra of TBDMS-derivatives of ascorbate and dehydroascorbate have been described earlier by Deutsch and Kolhouse [20]. The mass spectrum of TBDMS-ascorbate shows no obvious molecular ion at m/z 632 (Fig. 1A). Loss of a methyl group results in an ion at m/z 617, and for TBDMS-derivatives a characteristic loss of a *tert.*-butyl group from one of the four TBDMS-derivatized hydroxyl groups, generates an $[M-57]^+$ ion at m/z 575, which is also the base peak. Another prominent fragment can be observed at m/z 443, corresponding to a loss of a *tert.*-butyl group from one of the four TBDMS-groups and a loss of one TBDMS-OH.

No obvious molecular ion at m/z 402 can be observed in the EI-mass spectrum of TBDMS-dehydroascorbate (Fig. 2A). An $[M-15]^+$ ion at 387 of relatively low abundance, corresponding to a loss of a methyl group from one of the two TBDMS-derivatized hydroxyl groups can be observed. Base peak is the $[M-57]^+$ ion at m/z 345, corresponding to a loss of a *tert.*-butyl group from one of two TBDMS-derivatized hydroxyl groups. Full-scan spectra of ascorbate and dehydroascorbate were obtained from TCA extracts of Scots pine needles derivatized with TBDMS (Fig. 1B, Fig. 2B). The spectra were almost

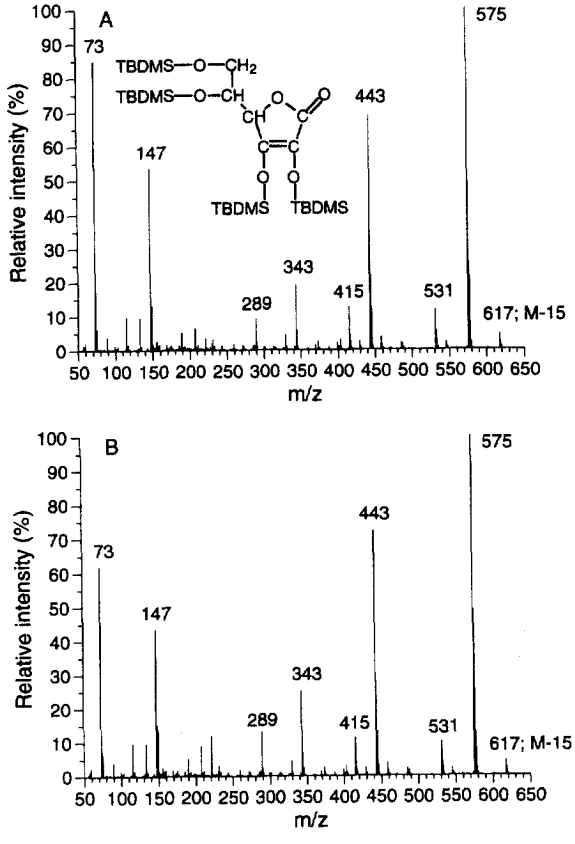

Fig. 1. Electron impact mass spectra of TBDMS-ascorbate standard (A) and from an extract of *Pinus sylvestris* (B).

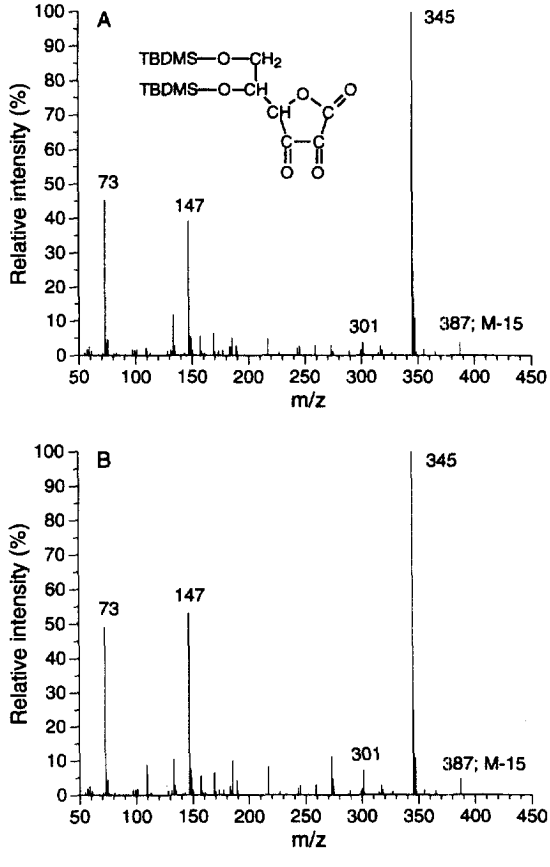

Fig. 2. Electron impact mass spectra of TBDMS-dehydroascorbate standard (A) and from an extract of *Pinus sylvestris* (B).

identical with the spectra of the standard compounds (Fig. 1A, Fig. 2A), clearly showing their presence in the extracts.

Quantitative analysis of ascorbate and dehydroascorbate was performed with HR-SIM. Ascorbate and dehydroascorbate were analysed in separate GC–MS analysis due to the combination to the difference in amounts and sensitivity, resulting in difficulties in tuning the mass spectrometer for both compounds in the same GC–MS run. However, it is theoretically possible to run both compounds in one GC–MS run. For the quantification of ascorbate, two characteristic ions and two $^{13}C_1$-labeled analogues were recorded. Analysis of a Scots pine extract showed that both ions are suitable for quantitative measurements since interfering substances could not be detected (Fig. 3). The quantification of dehydroascorbate was similarly based on the two characteristic ions and two ^{13}C-labeled analogues. Although either of the ion pairs could be used for the measurements, in practice the ion 345.1189 and its analogue 346.1189 were chosen for the quantification of dehydroascorbate as the 387.1659 ion and its analogue were of lower abundance. As in ascorbate analysis, no interfering substances could be detected near the dehydroascorbate peak (Fig. 4).

Calibration curves were recorded from 0.625 ng to 9.375 ng ascorbate/dehydroascorbate with a fixed amount of $^{13}C_1$ascorbate/dehydroascorbate as the internal standard (Figs. 5 and 6). The calibration curves are hyperbolic. The regression coefficients were in all cases between 0.9985 and 0.9993. The shapes of the curves depend on the number of labeled atoms in the internal standard. Since there is only one ^{13}C atom in the internal standard, the effect of the natural isotope abundance on the linearity is rather large [21,22]. As a consequence, for calculations of endogenous amounts of ascorbate/dehydroascorbate, a non-linear function were used. The variation in terms of R.S.D. of five replicate injections of 0.625 ng and 9.375 ng ascorbate and dehydroascorbate the GC–MS was less than 1% (Table 1).

When different extraction media including TCA (trichloroacetic acid, 5%), HCl (0.1 *M*) and $HClO_4$ (0.1 *M*) were tested, only the extraction with TCA was found to be suitable for both ascorbate and dehydroascorbate analysis (Table 2). Even though

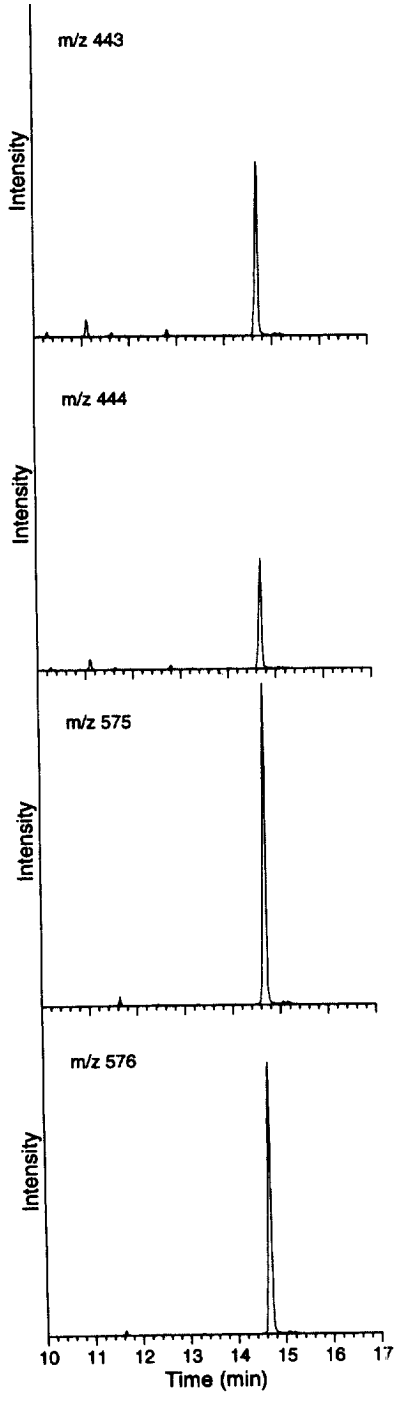

Fig. 3. Mass chromatogram for TBDMS-ascorbate detected in a plant extract of *Pinus sylvestris* by HR-SIM.

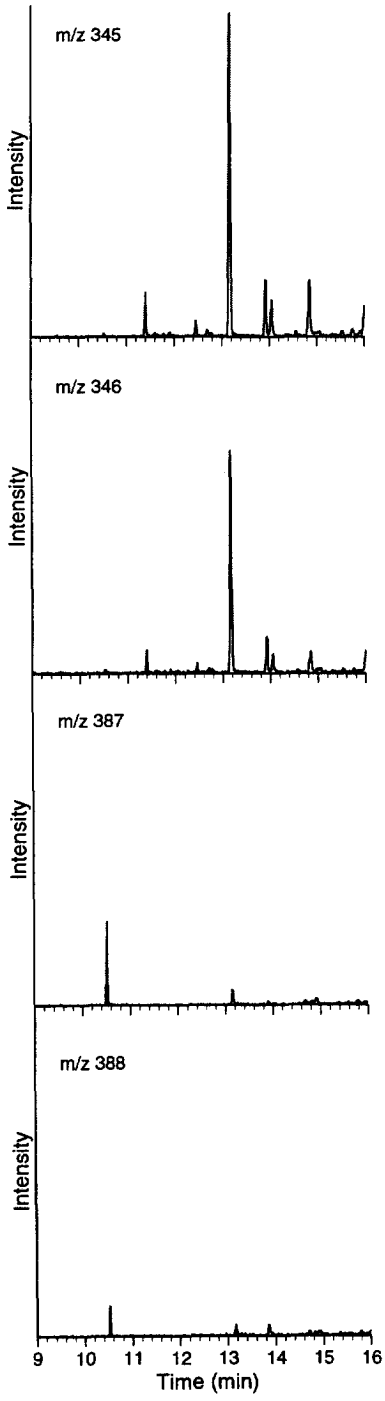

Fig. 4. Mass chromatogram for TBDMS-dehydroascorbate detected in a plant extract of *Pinus sylvestris* by HR-SIM.

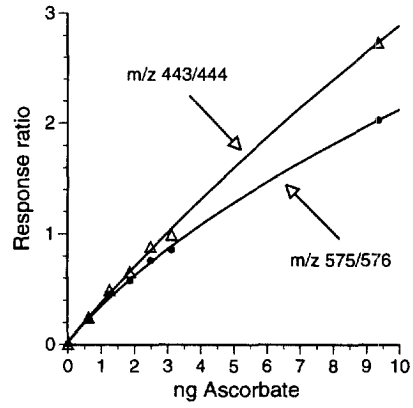

Fig. 5. Calibration curves for TBDMS-ascorbate.

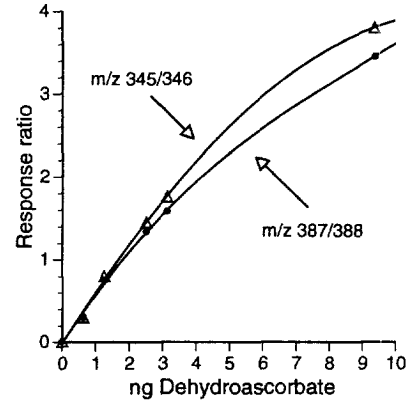

Fig. 6. Calibration curves for TBDMS-dehydroascorbate.

Table 1
Precision of ascorbate and dehydroacsorbate analysis by GC–MS

Substance	R.S.D. (%) for measured amount	
	0.625 ng	9.375 ng
Ascorbate	0.6	0.9
Dehydroascorbate	0.8	0.9

Standard mixtures of 0.625 and 9.375 ng ascorbate and dehydroascorbate together with a fixed amount of [$^{13}C_1$]ascorbate and [$^{13}C_1$]dehydroascorbate were analysed as TBDMS derivatives, and the relative standard deviation (R.S.D.) of the integrated area ratio for five replicate injections was calculated.

HCl (0.1 *M*) could be used in ascorbate measurements the dehydroascorbate could not be detected using this extraction medium. The precision of the whole quantification procedure, extracting with 5% TCA, was high for ascorbate ($n=11$, R.S.D.=4%)

Table 2
Effect of different extraction media on amounts of ascorbate (AsA) and dehydroascorbate (DAsA) detected in *Pinus sylvestris* Needle samples

	AsA	DAsA	AsA/(AsA+DAsA)
TCA (5%) (n=11)	5.12±0.202	0.393±0.040	0.929±0.007
HCl (0.1 M) (n=4)	4.43±0.624	n.d.	n.d.
$HClO_4$ (0.1 M) (n=4)	n.d.	n.d.	n.d.

The values are given as μmol (g fresh wt.)$^{-1}$ (mean±S.D.).
n.d.: not detected.

but lower for dehydroascorbate (R.S.D.=10%) (Table 2). However, when calculating the redox ratio of ascorbate/(ascorbate+dehydroascorbate) the reproducibility of the method was very high (R.S.D.= 1%).

The established GC–MS methodology described here was used for comparing ascorbate and dehydroascorbate levels in actively growing needles (from greenhouse-cultivated plants) and dormant needles (from plants in the field) collected in the late winter. The dormant needles showed significantly higher content of both ascorbate and dehydroascobate (Table 3). Also, the redox ratio of ascorbate was significant lower (P<0.05) in the dormant needles, although the absolute difference in the redox ratio was only approximately 1%; indicating the high level of reproducibility of the methodology.

4. Discussion

The main priority during analysis of ascorbate and dehydroascorbate in complex matrices with numerous potentially interfering substances, such as biological tissues, is to stabilize the compounds during extraction and subsequent analysis [12–14]. Small changes, especially in the amount of dehydroascorbate, normally of low abundance compared with ascorbate in biological tissues [2,5], will have a great impact on the derived of redox state of ascorbate [13,23,24]. Therefore both losses and conversions of these substances have to be prevented, or accounted for during extraction and analysis. The major advantage with GC–MS is the very high specificity of the analysis which, combined with the isotope dilution technique, results in highly accurate measurements. By detection of two ion pairs it is possible to check the accuracy of the analysis by calculating the measured amounts using both ion pairs. Although there are examples of sensitive analysis of biologically important compounds with fluorescence or UV detection, the accuracy of these methods can in some cases be questioned, as they are not methods with high specificity [14]. Analysis of complex biological matrices with these methods can result in inaccurate measurements due to the presence of interfering substances.

Table 3
Comparison of ascorbate (AsA) and dehydroascorbate (DAsA) in *Pinus sylvestris* needles growing outdoors during late winter (dormant) and in a greenhouse (actively growing)

	AsA	DAsA	AsA/(AsA+DAsA)
Dormant	4.924±0.287	0.378±0.023	0.929±0.003
Active	3.537±0.350	0.230±0.033	0.939±0.008

The needles were collected from current year needles from four different plants (mean±S.D.). The values are given as μmol (g fresh wt.)$^{-1}$.

GC–MS has become a widely used method for analysis of all sorts of biological compounds due to the benchtop quadrupole instruments that are available to a relative low cost. However, when analysis of complex matrices as plant extracts are to be performed, low resolution MS obtained results can be inaccurate. In our study we tried to analyse the semi-purified extract by low resolution GC–MS but the result was not acceptable due to interfering substances (data not shown). By using a double-focusing high resolution mass spectrometer (HR-MS) the selectivity and sensitivity of the analysis was increased compared to the low resolution measurements and the problems with interfering substances was overcome. The extremely high specificity of the HR-SIM analysis presented here, is linked to very high sensitivity; the limits in the detection of ascorbate and dehydroascorbate being approximately

0.5 pg (2.8 fmol) and 5 pg (14 fmol), respectively. The analysis of dehydroascorbate was approximately 10 times less sensitive. HPLC–UV spectrophotometric determination in serum reportedly has a maximal sensitivity of 10 pmol (2 ng) [18] and HPLC combined with coulometric detection gives even lower than 10 pmol in practical applications for ascorbate detection [25]. With the sensitivity of the GC–MS HR-SIM, a very specific area within a leaf or other plant segment can be analyzed with high accuracy. Thus this technique opens new perspectives for investigation of distribution patterns of these substances.

For interpretation of quantitative data and for optimizing experimental design, an estimate of the error associated with the quantification of the experimental material under investigation is valuable. The variation related to the GC–MS was less than 1% and the whole quantification procedure involved a R.S.D. of 4% and 10% for ascorbate and dehydroascorbate, respectively. This additional variation reflects weighing and pipetting errors. When the redox ratio of ascorbate/(ascorbate+dehydroascorbate) was analyzed, this kind of error was reduced and a very low R.S.D. was found (1%). This will make it possible to determine the redox state of ascorbate with a very high precision in different plant tissues and cell organelles under different environmental conditions.

Freezing and thawing of biological samples before being extracted by stabilizing media has been reported to degrade ascorbate [20]. Therefore it is very important, for every sample set, to test if there is any reduction/oxidation of ascorbate/dehydroascorbate. In the present study this was tested by adding [$^{13}C_1$]ascorbate or [$^{13}C_1$]dehydroascorbate separately to the extraction medium during the 5% TCA extraction of Scots pine needles. No oxidation/reduction of ascorbate or dehydroascorbate was detected (data not shown). As there was no detected conversion of [$^{13}C_1$]ascorbate to [$^{13}C_1$]dehydroascorbate or vice versa, it is possible to add both of the internal standards of ascorbate and dehydroascorbate to the extraction media prior to the extraction of the needles. This procedure is different from that reported by Deusch and Kolhouse [20], who prepared borosilicate tubes with dried ascorbate isotope prior to analysis of plasma or serum. Furthermore, these authors used $^{13}C_6$ labels which are more expensive and more difficult to obtain than $^{13}C_1$ labels making the current assay more practical. An alternative way of checking for the inter-conversion of ascorbate/dehydroascorbate during extraction would be for to use one internal standard with more labeled atoms, e.g., [$^{13}C_6$]dehydroascorbate [20] together with the [$^{13}C_1$]ascorbate. Potentially interfering conversions of ascorbate to dehydroascorbate could then be detected by monitoring [$^{13}C_1$]dehydroascorbate and conversions of dehydroascorbate to ascorbate could be detected by monitoring [$^{13}C_6$]ascorbate. However, the established extraction method presented in this paper, gives excellent results with semipurified Scots pine extracts, even though conifers have been reported to be difficult plant tissues to extract due to rapid oxidation in samples resulting from their high phenolic and tannin content [26].

Needles collected from outdoor plants exposed to sunlight during April showed a higher ascorbate and dehydroascorbate content than needles collected from actively growing plants. When comparing the redox state of ascorbate a slightly, but significantly lower ratio was found in the dormant needles (emphasizing the high precision of measuring the redox state of ascorbate by GC–MS). The plants in the field have been described earlier as having indications of photoxidative stress, including changes in chlorophyll fluorescence characteristics, and a reduced amount of pigments [27]. If continued oxidation is applied to dehydroascorbate it is irreversible degraded to 2,3-diketogulonic acid and other five or less carbons [14]. This implies that other ascorbate oxidation products are important to analyse in oxidative stress experiments. The higher amount of ascorbate is consistent with earlier reports that conifers show a seasonal ascorbate variation with a higher amount of ascorbate during the winter than in the growing season [5,17]. Andersson et al. [17] also measured the dehydroascorbate, and found there was a decreased redox state of ascorbate in the winter. However, no variation was shown for the HPLC-analysis of dehydroascorbate, which was measured indirectly, and may reflect difficulties with the analysis.

Several reports refer to various chemical and chromatographic methods available for determining

ascorbate and dehydroascorbate. The HR-SIM methodology used gives a very high specificity and sensitivity and offers an advance in the ability to analyze simultaneously both ascorbate and dehydroascorbate in complex biological matrices such as plant extracts.

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References

[1] S. Lewin, Vitamin C: Its Molecular Biology and Medical Potential, Academic Press, New York, 1976, p. 5.
[2] O. Arrigoni, J. Bioenerg. Biomembr. 26 (1994) 407.
[3] K. Asada, in C.H. Foyer and P.M. Mullineaux (Editors), Causes of Photooxidative Stress and Amelioration of Defence Systems in Plants, CRC Press, Boca Raton, Ann Arbor, London, Tokyo, 1994, p. 77.
[4] F. Cordoba, J.A. Gonzalez-Reyes, J. Bioenerg. Biomembr. 26 (1994) 399.
[5] A. Polle and H. Rennenberg, in C.H. Foyer and P.M. Mullineaux (Editors), Causes of Photooxidative Stress and Amelioration of Defence Systems in Plants, CRC Press, Boca Raton, Ann Arbor, London, Tokyo, 1994, p. 199.
[6] H. Mehlhorn, M. Lenlandais, H.G. Korth, C.H. Foyer, FEBS Lett. 378 (1996) 203.
[7] M. Luwe, U. Heber, Planta 197 (1995) 448.
[8] C.L.M. Sgherri, B. Loggini, S. Puliga, F. Navari-Izzo, Phytochemistry 35 (1994) 561.
[9] I. Kullik, G. Storz, Redox Report 1 (1994) 23.
[10] W.W. Wells, D.P. Xu, J. Bioenerg. Biomembr. 26 (1994) 369.
[11] J.R. Cooke and R.E.D. Moxon in J.N. Counsell and D.H. Hornig (Editors), Vitamin C, Applied Science Publishers, London, 1981, p. 167.
[12] H.E. Sauberlich, M.D. Green and S.T. Omaye, in P.A Seib and B.M. Tolbert (Editors), Ascorbic Acid—Chemistry, Metabolism and Uses, American Chemical Society, Washington, DC, 1982, p. 199.
[13] P.W. Wasko, W.O. Harzell, M. Levine, Anal. Biochem. 181 (1989) 276.
[14] P.W. Washko, R.W. Welch, K.R. Dhariwal, Y. Wang, M. Levine, Anal. Biochem. 204 (1992) 1.
[15] M. Okamura, Clin. Chim. Acta 103 (1980) 259.
[16] S.A. Margolis, T.P. Davis, Clin. Chem. 34 (1988) 2217.
[17] J.V. Andersson, B. Chevone, J. Hess, Plant Physiol. 98 (1992) 501.
[18] G. Barja, A. Hernanz, Methods Enzymol. 234 (1994) 331.
[19] K. Kampfenkel, M.V. Montagu, D. Inzé, Anal. Biochem. 225 (1995) 165.
[20] J.C. Deutsch, J.F. Kolhouse, Anal. Chem. 65 (1993) 321.
[21] J.F. Pickup, K. McPherson, Anal. Chem. 48 (1976) 1885.
[22] B.N. Colby, M.W. McCaman, Biomed. Mass Spectrom. 6 (1979) 225.
[23] F.G. Hopkins, J.M.C. Morgan, Biochem. J. 30 (1936) 1446.
[24] B.S. Winkler, S.M. Orselli, T.S. Rex, Free Rad. Biol. Med. 17 (1994) 333.
[25] K.R. Dhariwal, P.W. Washko, M. Levine, Anal. Biochem. 189 (1990) 18.
[26] B. Nyman, Phytochemistry 24 (1985) 2939.
[27] S. Karpinski, G. Wingsle, B. Karpinska, J.-E. Hällgren, Plant Physiol. 103 (1993) 1385.