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Characteristic fragmentation patterns of trimethylsilyl and trimethylsilyl-oxime derivatives of plant disaccharides as obtained by gas chromatography coupled to ion-trap mass spectrometry

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ABSTRACT

The characteristic fragmentation pattern of six reducing and two non reducing type disaccharides – (neohesperidose, acuminose, sambubiose, rutinose, vicianose, primverose, and two arabinosyl-inositols) has been described. These saccharides have not been previously identified by on-line chromatographic techniques. Unambiguous specific characteristics of the TMS (oxime)s such as mass distribution, syn/anti oximes ratios and elution order proved to be associated with their reducing or non reducing character, with their aldosyl property and with the position of their *O*-glycosidic linkages. The practical utility of the mass fragmentation study of these rare disaccharides was demonstrated, at the first time, by the simultaneous, on-line identification and quantification of the acuminose, vicianose, primverose and arabinosyl-inositol contents of tea leaves, from green and black tea blends of Indian and Chinese origin.

1. Introduction

The role of β-glycosidase, in cell wall degradation, lignification and defense is of primary importance [1].

It is known [2] that certain, glycosides accumulating plants contain glycosidase enzymes separately from their substrates. Cell disruption, initiated by homogenization, terminates this separation resulting in the hydrolysis of glycosides into the corresponding saccharide and aglycone molecules.

In order to follow these enzymatic processes, mainly, the intact glycosides were analyzed by nuclear magnetic resonance (NMR) spectroscopy [3–8], both by the ^{13}C NMR [3,4] and by the ^{1}H NMR [5–8] versions. The various separations by different affinity chromatographic systems [9–13] were followed applying hydrolyses. The hydrolysis products were identified without derivatization by thin layer chromatography (TLC) [3–6], by high performance liquid chromatography [11] and as trifluoroacetylated derivatives by GC–MS [10].

This work presents results concerning the qualitative and quantitative analysis of some disaccharides released after enzymatic hydrolysis, using our previously reported on-line technique [14,15].

This study at the first time, involves, the detailed fragmentation pattern, retention properties, identification and quantification essay of eight disaccharides, like

(a) neohesperidose and rutinose, (acidic hydrolysis products of their glycosides, naringin and rutin),
(b) acuminose, sambubiose, vicianose, and primverose (hydrolysis products of diglycosidase enzymes, obtained in order of listing from the leaves of *Viburnum furcatum*, from the flowers of *Hibiscus sabdariffa*, from the seeds of *Vicia angustifolia*, from the roots of *Rubia tinctorum*, as well as,
(c) acuminose, vicianose, primverose and two arabinosyl-inositols, compounds in free form, from the leaves of *Thea sinensis*.

2. Experimental

2.1. Materials

Materials and reagents were all of analytical reagent grade.

Methyl alcohol, pyridine, hydroxylamine hydrochloride, acetonitrile were from Reanal (Budapest, Hungary), hexamethyldisilazane (HMDS) and trifluoroacetic acid (TFA) from Serva (Heidelberg, Germany). Neohesperidose (2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranose) and rutinose (6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranose) were obtained by acidic hydrolysis of their

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glycosides, naringin and rutin, both products of Sigma–Aldrich (St. Louis, MO, USA). Acuminose (6-*O*-*β*-D-apiofuranosyl-*β*-D-glucopyranose, enzymatic hydrolysis product of the leaves of *V. furcatum* Blume ex Maxim. (VF-2010-499), was gift of the Institute of Ecology and Botany (Hungarian Academy of Sciences, Vácrátot). Sambubiose (2-*O*-*β*-D-xylanopyranosyl-*β*-D-glucopyranose) was enzymatic hydrolysis product of the flowers of *H. sabdariffa* L. (HS-2010-500), was bought in a local drugstore. Vicianose (6-*O*-*α*-L-arabinopyranosyl-*β*-D-glucopyranose), and primverose (6-*O*-*β*-D-xylanopyranosyl-*β*-D-glucopyranose) were enzymatic hydrolysis products of the seeds of *V. angustifolia* L. (VA-2010-501) and that of the roots of *R. tinctorum* L. (RT-2010-502), both were collected in the Botanical Garden of L. Eötvös University, Budapest).

The source of arabinosyl-inositols, was different tea (*T. sinensis* L.) blends bought in a local drugstore: such as two black, Darjeeling Automnal (DA-2010-503) from India, Golden Yunnan (GY-2010-504) from China, and two green tea blends, Darjeeling Risheehat (DR-2010-505) from India, Yunnan Green (YG-2010-506) from China. (*Note:* the voucher species of plant and tea blend samples were deposited in the Department of Plant Anatomy, L. Eötvös University, Budapest Hungary; samples' indications in parentheses.)

2.2. Preparation of plant extracts and acidic hydrolysis of naringin and rutin

0.02 g lyophilized, pulverized and homogenized samples were extracted three times (2 mL + 2 mL + 1 mL) with 80% (v/v) methyl alcohol applying a reflux condenser (1 h). The combined supernatants were completed to 5.0 mL stock solutions. Aliquots (0.25–1.00 mL, weighed with analytical precision) were evaporated to dryness on a vacuum evaporator at 30–40 °C, in 2–4 mL screw capped vials. Hydrolysis of naringin and rutin was performed with 200 μL 2 M TFA, varying the time (5–120 min) and temperature (60–100 °C). Samples were dried on a vacuum evaporator, at 30–40 °C and analyzed, subsequently to their derivatization, by GC–MS.

2.3. Preparation of plant extracts for enzymatic hydrolysis

0.02 g plant samples were stirred with 0.5 mL distilled water at 40 °C, for 15, 30, 60 and 180 min. The sample was then dried on a vacuum evaporator at 30–40 °C and extracted according to Section 2.2. Dried samples were measured subsequently to their derivatization by GC–MS.

2.4. Preparation of the trimethylsilyl (oxime) ether derivatives

Various amounts of the stock solutions of untreated and/or hydrolyzed standards (rutin, naringin) and plant extracts were evaporated to dryness on a vacuum evaporator, at 30–40 °C, in 2–4 mL screw capped vials. The dry residues were treated with 250 μL hydroxylamine hydrochloride containing pyridine (2.5 g/100 mL), at 70 °C, for 30 min. Silylation was performed in the same vial with 450 μL HMDS + 50 μL TFA, and heated at 100 °C for 60 min. After dilution with HMDS (performed with analytical precision), 1 μL of the diluted solutions was injected into the GC–MS system.

2.5. Gas chromatography–mass spectrometry (GC–MS)

The instrument, the separation and the acquisition conditions were exactly the same as reported in paper [15].

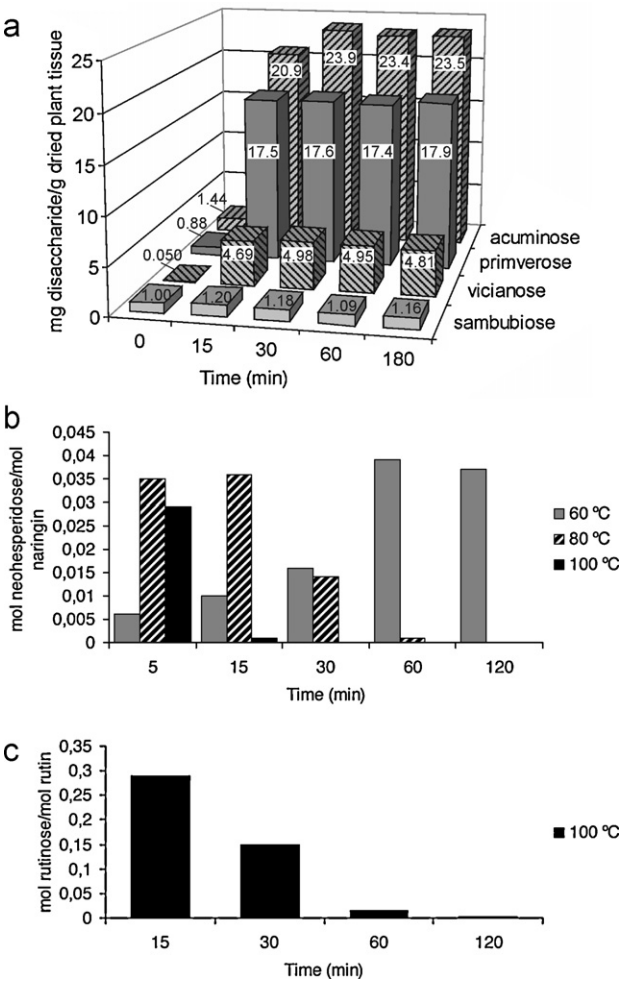

Fig. 1. (a–c) Hydrolysis studies of disaccharides based on the quantitation of their trimethylsilyl (TMS) (oxime) ether derivatives. (a) Enzymatic hydrolysis of plant extracts followed as a function of time, expressed in mg disaccharide/g dried plant tissue. (b and c) Acidic hydrolysis products of naringin (b) and rutin (c) depending on the time and temperature of hydrolyses, expressed as mol disaccharide/mol glycoside.

3. Results and discussion

3.1. Hydrolysis studies

Hydrolyses of the plant extracts with their corresponding diglycosidase enzymes show unambiguously (Fig. 1a) that

(a) in all cases investigated disaccharides are present also in unhydrolyzed samples, in free forms: their amounts, expressed as mg disaccharide/g dried plant tissue vary, in average, between ~1% (vicianose) and ~86% (sambubiose), calculated in the total of disaccharides determined after hydrolysis by diglycosidases,
(b) the release of disaccharides by enzymes proved to be quantitative within 15–30 min, and
(c) species remain stable up to 180 min.

TFA hydrolyses of naringin and rutin reveal considerably different stability of their disaccharide products (Fig. 1b and c), it means, instead of the stoichiometric 1 mol disaccharide/1 mol glycoside

(a) neohesperidose showed up as low as 0.04 mol/1 mol naringin obtained at 60 °C, after 60 min hydrolysis (Fig. 1b), while

Table 1
Relative intensities of the selective fragment ions of the trimethylsilyl-oxime and trimethylsilyl derivatives of plant disaccharides.

Disaccharides	t_R (min)	syn/anti oximes[a]	Selective fragment ions, m/z									
			259	273	319	343	349	358	363	433	448	538
			Relative intensities									
Neohesperidose-1A	18.41	4.0	–	1.44	11.7	–	–	0.5	0.69	–	0.46	0.20
Neohesperidose-1B	18.85		–	1.61	13.6	–	–	0.6	0.82	–	0.11	0.40
Acuminose-2A	18.63	3.4	20.0	–	0.83	–	0.07	0.22	–	–	0.93	1.24
Acuminose-2B	18.97		17.3	–	0.47	–	0.02	0.19	–	–	0.12	0.24
Sambubiose-3A	18.80	2.3	5.5	–	8.9	–	0.05	0.79	–	–	0.48	0.20
Sambubiose-3B	19.14		4.75	–	8.4	–	0.09	0.46	–	–	0.11	0.27
Rutinose-4A	18.94	3.2	–	4.11	1.77	–	–	0.73	4.55	–	1.04	1.89
Rutinose-4B	19.28		–	5.7	2.08	–	–	0.37	5.3	–	0.17	0.27
Vicianose-5A	19.06	3.9	19.9	–	1.29	–	0.21	0.49	–	–	0.69	0.84
Vicianose-5B	19.46		18.4	–	1.16	–	0.15	0.26	–	–	0.13	0.19
Arabinosyl-inositol-6	18.75	–	2.83	–	1.02	1.21	–	–	–	4.99	–	–
Arabinosyl-inositol-7	19.25	–	2.83	–	–	3.65	–	–	–	6.0	–	–
Primverose-8A	19.56	3.2	15.6	–	1.16	–	0.5	0.42	–	–	0.39	0.51
Primverose-8B	19.90		13.9	–	1.09	–	0.43	0.16	–	–	0.07	0.07

Indications: disaccharides' name and spectra are denoted as in Figs. 1 and 2.

[a] Response ratios of the syn and anti oximes (spectra – 1A, 1B; 5A, 5B and 8A, 8B correspond to the syn and anti oximes; derivatives 6 and 7 are arabinosyl-inositols with the same spectra); the huge TMS-sucrose is present in all chromatograms (t_R = 18.13 min).

(b) rutinose proved to be notably more stable: even at 100 °C, after 15 min hydrolysis time, as maximum amount, 0.28 mol rutionse/1 mol rutin was measured (Fig. 1c). In both cases satisfactory amounts for their mass fragmentation and retention characterization studies are detailed in Section 3.2 (Table 1 and Fig. 2).

3.2. Fragmentation/retention properties, identification and quantification study of rare disaccharides, performed by gas chromatography mass spectrometry

In addition to our earlier experiences [14,15], relating to the GC–MS mass fragmentation properties of primverose [14,15] and rutinose [15], as a new principle to the field, the list of rare, plant disaccharides, not available as standards, has been completed. Their simultaneous elution, as their TMS (oxime) ether derivatives (Fig. 2, traces a–g) repeatedly reveal [15], that all TMS-oxime derivatives of the reducing glycosides are consisting of two different moieties: one moiety of ring structure and one moiety of open chain structure.

(a) The moieties of ring structure, like all non reducing glycosides do provide the characteristic and corresponding selective fragment ions (SFIs), i.e.,
  – from the rhamnopyranosyl neohesperidose and rutinose, the mass m/z 273 (Table 1 and Fig. 2, traces a and d, spectra 1A and 4A; m/z 363 – 90 = 273) while
  – from all 'pentosyls', like acuminose, sambubiose, vicianose, arabinopyranosyl-inositols and primeverose, the mass m/z 259 (Table 1 and Fig. 2, traces b, c, e–g, spectra 2A, 3A, 5A, 6 and 8A; m/z 349 – 90 = 259) were obtained.
(b) The moieties of open chain structure, i.e., the TMS-oxime group containing ones (except arabinosyl-inositols) furnish, independent on the species of glycosidic linkage, the fragment of specific masses m/z 538 (Table 1 and Fig. 2, traces a–e, g, spectra 1A–5A, and 8A). This experience is characteristic to the TMS-oxime group containing moiety of the open chain structure: to our knowledge, it is still a novelty, not available in the Nist and Whiley libraries, either.
(c) The cyclic property of inositol shows up with the mass m/z 433 (m/z = 523 – 90), (Table 1 and Fig. 2, trace f, spectrum 6).

Note: it is to be indicated that the corresponding 1B–5B and 8B spectra are identical with their presented A counterparts, as well as, the spectra of the two arabinosyl-inositol derivatives are also identical (spectrum 6), as demonstrated with their SFIs in Table 1).

The specificity of the oxime formation of reducing, aldosyl type disaccharides has been repeatedly characterized by the ratios of their syn and anti oximes (A and B derivatives), which proved to be revealing to aldoses. The syn/anti ratio values of reducing disaccharides (Table 1, values in third vertical column) vary between 2.3 (sambubiose) and 4.0 (neohesperidose). The syn/anti ratio values typical to ketoses are less than 1 (fructose = 0.70, palatinose = 0.94 [16]).

As to the impact of the position of the glycosidic linkages this phenomenon is defined both by the retention properties and by the characteristic selective fragment ions of counterparts (Table 1). This means, that

(a) 2-*O*-glycosides (neohesperidose, sambubiose) are eluting before their corresponding 6-*O*-glycosides (rutinose, primverose), as well as
(b) 2-*O*-glycosides do provide twenty times higher abundance of the mass m/z 319 in comparison to the 6-*O*-glycosides. The mass m/z 319 originates from the fragment ion m/z 409, formed by the loss of one TMSOH group (m/z 90) (Table 1 and Fig. 2, traces a, c: neohesperidose, sambubiose). The structural stability of the C_3–C_6 skeleton can be attributed to the virtual existence of the CH–$(CHOTMS)_2$–CH_2OTMS radical (m/z 409).

3.3. Rare disaccharide content of the leaves of T. sinensis

Before going into details with our study, it is worth mentioning that during black tea processing [11], on the impact of the β-diglycosidase, along with aroma compounds, even disaccharides, like acuminose, vicianose and primverose were expected: however, by HPLC, (in addition to glucose, fructose, sucrose, raffinose and arabinosyl myo-inositol), these rare disaccharides were not found [11].

Results of the analysis of four tea blends, based without exception on the evaluation of their SFIs m/z 259, are summarized in Table 2. These data show the practical utility of this study. Rare disaccharides, expected products of the black tea manufacturing process (acuminose, vicianose, primverose), or present initially in free form (arabinosyl-inositols), have been identified and quantitated in a single analysis, at the first time.

The obtained results showed that the studied black tea samples contained more amounts of acumionose, vicianose and primverose than the green samples.

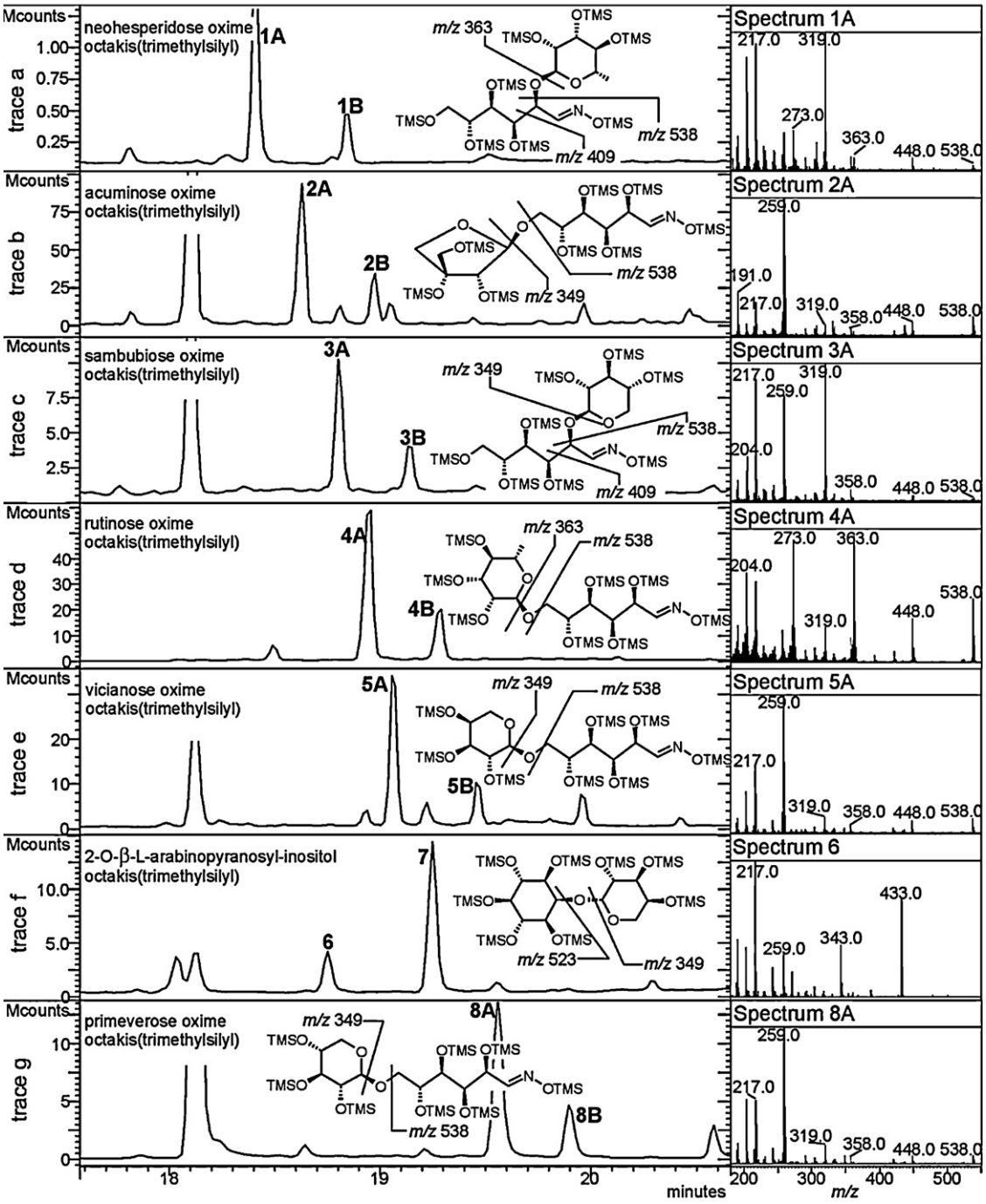

Fig. 2. Elution profile, mass spectra, and fragmentation patterns of the TMS (oxime) ether derivatives of neohesperidose (trace a, spectrum 1A), acuminose (trace b, spectrum 2A), sambubiose (trace c, spectrum 3A), rutinose (trace d, spectrum 4A) vicianose (trace e, spectrum 5A), primverose (trace g, spectrum 8A), and that of the TMS derivatives of arabinosyl-inositols (trace f, spectrum 7). Detailed retention properties and fragmentation pattern analysis are shown in Table 1.

Table 2
Disaccharides in tea leaves obtained as trimethylsilyl-oxime and trimethylsilyl derivatives, calculated on the fragment ions *m*/*z* 259.

Compounds	Disaccharides (mg/g dry tea leaves)			
	Black blends		Green blends	
	Golden Yunnan	Darjeeling automnal	Yunnan green	Darjeeling Risheehat
Acuminose-2A + 2B	0.017	0.024	–	0.0041
Vicianose-5A + 5B	0.018	0.011	–	0.0050
Primverose-8A + 8B	0.31	0.45	0.038	0.055
Arabinosyl-inositol-6	1.21	2.29	2.28	1.63
Arabinosyl-inositol-7	5.0	6.8	7.41	6.02

Indications as in Fig. 2 and Table 1.

The results also showed that the arabinosyl-inositol content vary between 6.2 mg/g and 9.2 mg/g, in agreement with the tentative literature data (≈8 mg/g), obtained by ^{13}C NMR spectroscopy [4].

4. Conclusion

1. Identification and quantification of rare plant disaccharides (acuminose, sambubiose, primverose, vicianose, neohesperidose, rutinose and arabinosyl-inositols) were performed on the basis of their selective fragment ions, obtained by gas chromatography mass spectrometry as their trimethylsilyl (oxime) ether derivatives.
2. It has been repeatedly proved that basic difference exists between the reducing and non reducing disaccharides, in terms of their special fragmentation pattern properties. It means, all TMS-oxime derivatives of the reducing disaccharides are consisting of two different moieties: one moiety of ring structure and one moiety of open chain structure: providing specific, selective fragment ions suitable for their identification and quantification purposes.
3. As novelty to the field, it was described at the first time,

(a) both the retention order
(b) and the selective fragment ion characteristics of rare plant disaccharides as their TMS (oxime) ether derivatives: resulted in their simultaneous identification and quantification in a single chromatographic run.
(c) The relative abundance of selective fragment ions, due to the position of the *O*-glycosidic linkages, was also a new experience: it was shown that 2-*O*-glycosides provide twenty times higher abundance of the mass *m*/*z* 319 in comparison to the 6-*O*-glycosides.

4. Results of basic studies have been utilized in the on-line determination of the rare disaccharide content of the extracts of two green and two black tea blends. Data proved that the

(a) arabinosyl-inositol type disaccharides are the main constituents; in black and green tea leaves, in total, 6.2–9.7 mg/g dried tea leaves have been measured.
(b) acuminose, vicianose and primverose, characteristic products of tea aroma processing, in black tea leaves were found in considerably higher concentrations (0.011–0.45 mg/g dried tea leaves) in comparison to their amounts determined in green tea leaves (0.0–0.055 mg/g dried tea leaves).

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