Table II. Analysis of Test Samples for FX,[a] DON, and NIV

| grain | country of origin | anal., ng/g DON | NIV |
|---|---|---|---|
| wheat | England | 188 | 103 |
| wheat | England | 20 | 78 |
| wheat | England | 20 | 232 |
| wheat | England | 41 | 30 |
| rye | Japan | nd[b] | 58 |
| wheat | Japan | nd | 63 |
| polished barley | Japan | 20 | 204 |
| barley | Japan | 110 | 347 |
| barley | U.S. | 618 | nd |
| mixed feed | U.S. | 25300 | nd |

[a] No FX was detected in any of these grains. [b] nd = not detected.

silica gel TLC plate was prewashed with the second developer.

To evaluate the applicability of the method to naturally contaminated grains, 10 laboratory samples of wheat and barley received from England and Japan were analyzed for FX, DON, and NIV. These laboratory samples had been previously analyzed in several laboratories and were reported to contain NIV and/or DON (Gilbert, 1985; Ueno, 1985). Results of the analyses are shown in Table II. No FX was detected. The identities of DON and NIV were confirmed by GC/MI/FTIR spectrometry (Mossoba et al., 1986). This multimycotoxin TLC method can be used to survey simultaneously for FX, DON, and NIV in corn, wheat, or barley and should be applicable to other grains.

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**Registry No.** FX, 23255-69-8; DON, 51481-10-8; NIV, 23282-20-4.

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# Simultaneous Determination of Heavy Metals in *Chlorella* and Tea Leaves by High-Performance Liquid Chromatography

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Nickel, zinc, and copper in standard reference *Chlorella* and tea leaves were simultaneously determined by high-performance liquid chromatography (HPLC). The samples were ashed with mineral acids, and then the metals were extracted in chloroform as hexamethylenedithiocarbamate (HMDC) chelates. The metal chelates were separated with HPLC using a $C_{18}$ column and detected at 260 nm (*Chlorella*) or 290 nm (tea leaves). Interference of manganese in the sample was removed with use of L-ascorbic acid. Results showed good agreement with certified values of the samples. The standard deviations based on 6-12 replicate determinations were 1.1-7.2%.

Heavy metals in plant samples are frequently determined by atomic absorption spectrometry (AAS) after undergoing an appropriate ashing procedure. Inductively coupled plasma atomic emission spectrometry (ICP) is also used if multielement analysis is necessary.

Simultaneous determination of heavy metals by HPLC has been studied in recent years (Willeford and Veening, 1983; O'Laughlin, 1984; Nickless, 1985). The HPLC ena-

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bles multielement determination, requires less expensive instrumentation, and is not as complicated to operate as AAS and ICP.

In HPLC procedures for heavy metals, dithiocarbamates have been used as chelating agents (Edward-Inatimi, 1983; Bond and Wallace, 1984; Smith et al., 1985). Unfortunately, there have been very few applications of this procedure to samples other than water (Edward-Inatimi, 1983; Ichinoki et al., 1984; Ichinoki and Yamazaki, 1985). A pretreatment method (ashing or extraction) suitable for one type of sample is not always suitable for another due to different matrices present in the samples. In fact, a large amount of manganese interfered with HPLC determination of heavy metals when a previous method (Ichinoki and Yamazaki, 1985) was used.

The purpose of the study presented here was to develop a suitable ashing and extraction procedure to be used with HPLC determination of heavy metals for the plant samples containing a large amount of manganese.

## EXPERIMENTAL METHODS AND PROCEDURES

**Reagents and Standard Solutions.** Standard reference materials (*Chlorella*, tea leaves) were obtained from the National Institute for Environmental Studies (NIES, Tsukuba, Ibaraki, Japan). The samples were dried at 85 °C for 4 h before use. Nitric acid, perchloric acid, hydrofluoric acid, and ammonia water used in this experiment were a high-purity grade prepared for heavy-metal analysis (Wako Pure Chemical Industries Ltd., Osaka, Japan). Heavy-metal contaminants in ammonium citrate solution (1 M, pH 9) were removed by extracting with a dithizone–chloroform solution. Hexamethyleneammonium hexamethylenedithiocarbamate (HMA-HMDC) was synthesized according to a procedure described in a previous paper (Ichinoki et al., 1984). All solvents used were of LC grade. The other reagents were of analytical grade.

The column packing used was Cosmosil 5 $C_{18}$ (5 $\mu$m ODS, Nakarai Chemicals Ltd., Kyoto, Japan).

The standard solution for tea leaves analysis had 350 ng/mL of Ni, 39 ng/mL of Pb, 1700 ng/mL of Zn, and 350 ng/mL of Cu in 0.1 M hydrochloric acid. Five milliliters of the standard solution contained approximately the same amount of metals as in 250 mg of tea leaves. A similar standard solution was prepared for *Chlorella*.

**Apparatus.** The liquid chromatograph used in this study consisted of a Model KHP-010 pump (Kyowa Seimitsu Co. Ltd., Tokyo, Japan), a Model UVIDEC-100-III variable-wavelength detector (Japan Spectroscopic Co., Tokyo, Japan), and a Model C-R1B data processor (Shimadzu Co., Kyoto, Japan). Eluted metal chelates were detected at 260 nm (*Chlorella*) or 290 nm (tea leaves, sensitive for nickel; Ichinoki et al., 1984). The stainless steel column (4.6 mm (i.d.) × 250 mm) packed with Cosmosil 5 $C_{18}$ was immersed in a constant-temperature water bath (40.0 ± 0.1 °C).

**Ashing Procedure.** (*A*) *Ashing Procedure for Chlorella.* In a 50-mL glass conical beaker, 250 mg of *Chlorella* was mixed with 5 mL of $HNO_3$–$HClO_4$ mixture (1:1, v/v). The beaker was then covered with a watch glass and allowed to stand overnight. Contents of the beaker were heated gradually on a hot plate (to 50 °C in 10 min, from 50 to 75 °C in 10 min, from 75 to 100 °C in 10 min, from 100 to 150 °C in 10 min) for 50 min at 150 °C. After cooling, 5 mL of perchloric acid was added and the contents were heated at 200 °C until a colorless clear solution was obtained. The watch glass rinsed with deionized water, the rinse water was added to the beaker, and the entire contents were evaporated to about 3 mL. Again, 5 mL of perchloric acid was added slowly along the inside beaker wall after cooling, and the digestate was evaporated to dryness at 100–150 °C.

(*B*) *Ashing Procedure for Tea Leaves.* The ashing procedure for tea leaves was slightly different. After the second addition of perchloric acid, the digestate was transferred to a polyfluoroethylene (Teflon) beaker; 5 mL of hydrofluoric acid was then added and the resultant mixture evaporated to dryness at 150 °C. Blank tests were carried out to eliminate contamination from the acid and the glassware used for ashing.

**Extraction Procedure for Heavy Metals in Biological Samples.** The ash residue obtained by the above procedure was dissolved in 5 mL of 1 M nitric acid and the resultant solution mixed with 35 mL of deionized water and 10 mL of 1 M ammonium citrate solution (pH 9). Two grams of L-ascorbic acid (vitamin C) was dissolved in the mixture. This step was not necessary if the sample was not high in manganese, such as *Chlorella*. After the pH was adjusted to 9.0 with 25% ammonia water, the solution was transferred to a 100-mL glass separatory funnel. The solution was diluted to 60 mL with deionized water, and then 3.0 mL of 0.01 M HMA-HMDC and 1.0 mL of chloroform were added. The contents were shaken for 15 min and then allowed to stand for 3 min. Ten microliters of the chloroform phase was used for HPLC analysis. Under these conditions, nanogram/milliliter levels of Cd, Ni, Pb, Zn, Cu, Hg, Co, and Bi may be extracted simultaneously. Details of the extraction of HMDC chelates into chloroform were described by Ichinoki et al. (1984).

**HPLC Conditions.** Conditions for HPLC in metal determinations were as follows: (1) column, Cosmosil 5 $C_{18}$; (2) eluent; methanol–water–chloroform–0.01 M HMA-HMDC (76:16.5:6:1.5); (3) flow rate, 0.8 mL/min; (4) detection wavelength, 260 nm (for *Chlorella*) and 290 nm (for tea leaves); (5) injection volume, 10 $\mu$L; (6) detector response, 0.08 AUFS; (7) attenuation of data processor, 3–5 (8–32 mV). In order to determine nickel in tea leaves sensitively, 290 nm was used (Ichinoki et al., 1984).

The eluent reservoir was cooled in an ice bath (Ichinoki et al., 1984). These conditions permitted base-line separation of Cd–, Ni–, Pb–, Zn–, Cu–, Hg–, Co–, and Bi–HMDC chelates (Ichinoki and Yamazaki, 1985).

Fifty milliliters of a mixture of methanol–water–chloroform (76:18:6) (flowed through the normal path of the HPLC apparatus: pump–injector–column–detector) was used to remove HMA-HMDC from the column packing after the analyses were completed each day.

**Calibration Curves.** The blank (5 mL of 1 M nitric acid to dissolve residues of the acid blank, 35 mL of deionized water, and 10 mL of 1 M ammonium citrate solution (pH 9) in a blank beaker) and standard (5 mL of 1 M nitric acid, 10 mL of mixed standard solution, 25 mL of deionized water, and 10 mL of 1 M ammonium citrate solution, pH 9, in another blank beaker) were adjusted to pH 9 with 25% ammonia water and transferred to separatory funnels for extraction. Each solution was diluted to 60 mL with deionized water. Volumes of 3.0 mL of 0.01 M HMA-HMDC and 1.0 mL of chloroform were added to the separatory funnel, and the contents were shaken for 15 min and then allowed to stand for 3 min. Ten microliters of the chloroform phase was injected to the HPLC, and peak heights of the HMDC chelates were recorded. Three blank and standard solutions were used for preparing the calibration curves of heavy metals.

## RESULTS AND DISCUSSION

**Examination of Masking Agents for Manganese.** Manganese(II) was also extracted in chloroform as Mn-

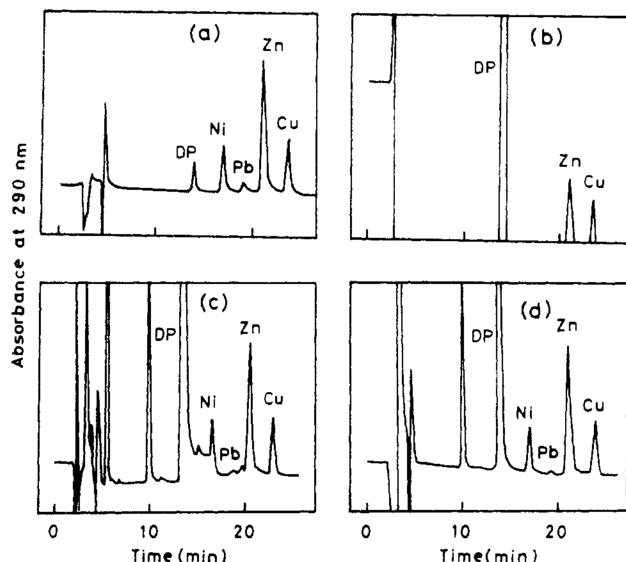


**Figure 1.** HPLC chromatograms of the effect of hydroxylamine hydrochloride and vitamin C on reducing Mn interferences in the determination of Ni–, Pb–, Zn–, and Cu–HMDC chelates in chloroform extracts from digestate of tea leaves. Concentration of metal in 50-mL aqueous phase: Ni, 35 ng/mL; Pb, 3.9 ng/mL; Zn, 170 ng/mL; Cu, 35 ng/mL. Key: (a) normal condition; (b) Mn (15 μg/mL) and Fe (10 μg/mL) present; (c) 2 g of $NH_2OH{\cdot}HCl$ added to a sample similar to (b); (d) 2 g of vitamin C added to a sample similar to (b); DP, decomposition product of HMA-HMDC.

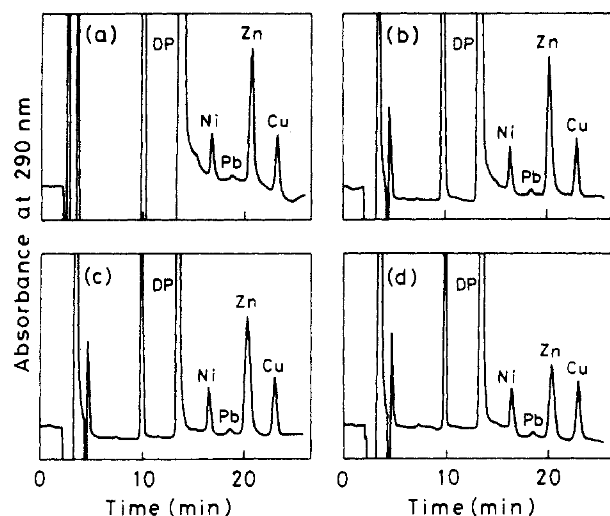


**Figure 2.** HPLC chromatogram of the effects of vitamin C on reducing Mn interferences in the determination of Ni–, Pb–, Zn–, and Cu–HMDC chelates in chloroform extracts from digestates of tea leaves. Vitamin C added: (a) 0.5 g; (b) 1.0 g; (c) 2.0 g; (d) 10.0 g. Conditions were similar to those reported in Figure 1.

$(HMDC)_3$. This chelate was not stable in chloroform phase and reversed-phase eluent. When a large amount of the chelate was extracted in chloroform, the base line of chromatogram was disturbed severely. To prevent the interference of manganese, chemical reductants must be used as masking agents. Several reductants, such as hydroxylamine hydrochloride, sodium thiosulfate, sodium sulfite, vitamin C, were tested. Both hydroxylamine hydrochloride and vitamin C were effective as masking agents for manganese.

Figure 1 shows the chromatograms of normal sample (a), of Mn interference (b), of the hydroxylamine hydrochloride treated sample (c), and of the vitamin C treated sample (d). Concentration of Mn (15 μg/mL) corresponds to 3000 μg/g and that of Fe (10 μg/mL) to 2000 μg/g in a 250-mg

**Table I. Certified or Reference Values (on a Dry-Weight Basis) of Constituent Elements in *Chlorella* and Tea Leaves**

| element | *Chlorella*, % | tea leaves, % | element | *Chlorella*, μg/g | tea leaves,[a] μg/g |
|---|---|---|---|---|---|
| K | 1.24 ± 0.06 | 1.87 | Fe | 1850 ± 100 | 34.1 |
| P | 1.7 | 0.37 | Al | | 775 |
| Ca | 0.49 ± 0.03 | 0.33 | Mn | 69 ± 5 | 690 |
| Mg | 0.33 ± 0.02 | 0.15 | Zn | 20.5 ± 1.0 | 34.1 |
| | | | Cu | 3.5 ± 0.3 | 7.1 |
| | | | Ni | | 6.5 |
| | | | Pb | 0.60 | 0.81 |
| | | | Cd | 0.026 | 0.032 |
| | | | Co | 0.87 ± 0.05 | |
| | | | Sr | 40 ± 3 | 3.7 |

[a] 34.1 μg/g by AAS, 32.0 μg/g by ICP. The values indicate certified values for *Chlorella* and reference values for tea leaves.

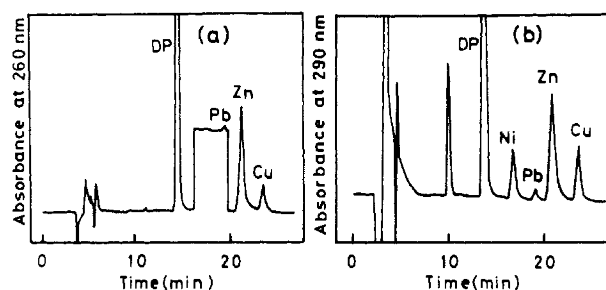


**Figure 3.** Analytical chromatogram of *Chlorella* (a) and tea leaves (b).

biological sample. The effect of vitamin C was further investigated with 0.5–10.0-g additions of vitamin C under condition (d). The results are shown in Figure 2. Although vitamin C was effective as a masking agent, a large amount of vitamin C (>3 g) resulted in a low recovery of zinc. For satisfactory metal recovery (97–103%), the amount of vitamin C must be kept to a minimum. Two grams of vitamin C was adequate to mask about 3000 μg/g of manganese in a 250-mg sample.

Because the Mn chelate in the chloroform phase decomposes rapidly with time at elevated temperature, allowing the chloroform phase to stand at room temperature before HPLC analysis is recommended for the samples containing small amount of manganese (less than about 100 μg/g in a 250-mg sample).

**Determination of Ni, Zn, and Cu in *Chlorella* and Tea Leaves.** The linear range of the calibration curve prepared from standard solution is from 1 to 1000 ng/mL (0.2–200 μg/g in a biological sample for a 250-mg sample). However, the blank values for determining heavy metals in biological samples were high because of contamination from the ambient environment at ashing step, glassware, and vitamin C reagent. The linear working range for a 250-mg sample was, therefore, 1-200 μg/g. Interestingly, Ni, Pb, Cd, and Co in *Chlorella* and Pb, Cd, and Co in tea leaves were too low to determine by the present method (Table I). Mercury and bismuth were not detected in the samples.

Analytical results for Ni, Zn, and Cu were summarized in Table II and analytical chromatograms shown in Figure 3. Table II shows that concentrations of Ni and Cu in tea leaves obtained by ashing procedure with $HNO_3$–$HClO_4$–HF (B) were slightly higher than those with $HNO_3$–$HClO_4$ (A) while Zn was slightly lower than that of (A).

Vitamin C was not used for *Chlorella* because Mn concentration was low (Table I). Figure 3b showed a stable base line in spite of the high manganese concentration (690 μg/g) in the tea leaves sample. This results indicated that

**Table II. Analytical Results of *Chlorella* and Tea Leaves**[a]

| sample | metal | found, μg/g | cv, % | certified values, μg/g |
|---|---|---|---|---|
| *Chlorella* | Zn | 20.55 ± 0.24 | 1.1 | 20.5 ± 1.0 |
| | Cu | 3.50 ± 0.05 | 1.4 | 3.5 ± 0.3 |
| tea leaves (A)[b] | Ni | 6.28 ± 0.18 | 2.9 | 6.5 |
| ($HNO_3$ + $HClO_4$)[c] | Zn | 34.5 ± 2.5 | 7.2 | 34.1 |
| | Cu | 6.76 ± 0.26 | 3.8 | 7.1 |
| tea leaves (B)[b] | Ni | 6.34 ± 0.39 | 6.1 | 6.5 |
| ($HNO_3$ + $HClO_4$ + HF)[c] | Zn | 32.6 ± 1.6 | 4.9 | 34.1 |
| | Cu | 7.03 ± 0.24 | 3.4 | 7.1 |

[a] The values of the tea leaves are reference values. [b] The standard deviation (cv) was based on 6–12 replicate determinations including ashing, extraction, and HPLC steps. $N$ = 6 for *Chlorella* and tea leaves (A). $N$ = 12 for tea leaves (B). [c] Acids used in wet ashing method.

vitamin C was an effective masking agent for Mn in this biological sample.

In general, the results of *Chlorella* and tea leaves showed good agreement with the certified values reported by the NIES (Table I).

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# Structure–Activity Relationships of Cyclic and Acyclic Analogues of the Phytotoxic Peptide Tentoxin

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Structurally modified peptide analogues of the phytotoxin tentoxin demonstrated differences in biological activity as a result of changes in stereochemistry, amide bond N-methylation, and cyclic conformational constraint. Peptide analogues were prepared through a solution-phase peptide synthetic route. The bioactive properties of the compounds were determined in a lettuce seedling assay employed to measure chlorosis induction. A 12-atom ring of the peptide backbone and N-methylation at dehydrophenylalanine were required for full biological activity. Removal of the *N*-methyl group at alanine resulted in little change in chlorosis-inducing activity, but absence of both *N*-methyl groups at dehydrophenylalanine and alanine resulted in complete loss of activity in the analogues containing L-amino acids. A change in stereochemistry at alanine and leucine was used to evaluate biological activity upon conversion of cis–trans–cis–trans backbone conformation of the native sequence to cis–cis–cis–trans. Acyclic analogues having N-methylation at dehydrophenylalanine demonstrated low but significant chlorosis activity.

In recent years increasing attention has been given to small cyclic peptides demonstrating selective phytotoxic and growth-regulating activity in plants (Stoessel, 1981; Shimohigashi et al., 1978; Ueda et al., 1985; Walton et al., 1982). A number of these bioactive peptides display analogous structural moieties including the following: (1) cyclic, four amino acid containing sequences; (2) secondary amino acids (*N*-methylalanine and proline); (3) dehydro amino acids; and (4) alkyl and aromatic amino acid side chains. The non-host-specific cyclic tetrapeptide tentoxin (Figure 1) induces chlorosis in a broad range of plants (Fulton et al., 1965). The host-specific cyclic tetrapeptide AM toxin (Figure 2) that induces necrosis on apple leaves (Ueno et al., 1975) shares some of the similar structural features. In contrast the cyclic tetrapeptide *cyclo*(L-Pro-L-Val-L-Pro-L-Val-) and the D-Val-containing isomer show selective stem and root growth-regulating activity in rice seedlings (Ueda et al., 1985).

The present study focuses on tentoxin, a metabolite from the fungus *Alternaria alternata*, that has the sequence *cyclo*(-*N*-methylalanyl-L-leucyl-*N*-methyl-(*Z*)-dehydrophenylalanylglycyl-) (Figure 1) (Meyer et al., 1974a; Fulton et al., 1965). The peptide induces chlorosis in germinating seedlings of some dicotyledonous plants but not in corn, tomato, and members of the Cruciferae and Gramineae (Fulton, 1965; Templeton, 1967). Chlorosis, which is a diseased condition in plants characterized by yellowing, is determined by extracting tentoxin-treated seedlings with $Me_2SO$ and comparing measured chlorophyll levels with untreated controls. Early work attributed chlorosis to inactivation of the photosynthetic phosphorylation enzyme coupling factor 1 ($CF_1$) (Steele et al., 1976). Previous structure–function studies demonstrated that conformers of [D-MeAla[1]]-, [Sar[1]]-, and [Pro[1]]tentoxin were similar to native tentoxin in binding and inhibition of the $CF_1$ enzyme (Rich and Bhatnagar, 1978; Rich et al., 1980).

Recent evidence suggests that tentoxin-induced chlorosis and impairment of chloroplast development is mediated through inhibiting transport of the nuclear-coded protein polyphenoloxidase (PPO) into the chloroplast (Vaughn and Duke, 1984; Duke and Vaughn, 1982; Duke et al., 1982; Lax et al., 1985). Earlier studies reporting impairment of starch degradation in cabbage (Halloin et al., 1970) where no chlorosis was observed further pose the possibility of more

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