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# Quantitative Analysis of Roussin Red Methyl Ester in Pickled Vegetables[1]

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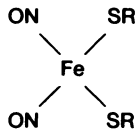
## ABSTRACT

In a study of the etiological factors in esophageal cancer, Roussin red methyl ester was isolated and identified in pickled vegetables of Linxian County, North China, where there is a high incidence of esophageal cancer. In this paper, a method is described for quantitative analysis of Roussin red methyl ester in pickled vegetables by gas chromatography-high-resolution mass spectrometry single-ion monitoring. The content of Roussin red methyl ester in pickled vegetables from Linxian has been found to be 0.1 to 4.5 mg/kg, and that from Beijing is below 0.005 mg/kg, which is the detection limit of the analytical method used. The marked difference between the contents might be one of the possible reasons for the difference in esophageal cancer incidence between the two regions.

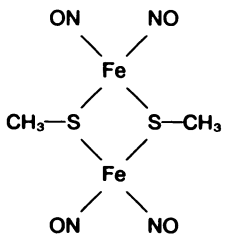
## INTRODUCTION

It is well known that many nitrosamines are carcinogenic toward some animal species, and some biologically important nitroso compounds have recently aroused special attention; but up to now only very little work on the nitroso compounds containing iron has been reported. Chiang *et al.* (2) and Woolum and Commoner (12) reported an electron spin resonance signal originating from some paramagnetic complex in the liver of rats fed with hepatic carcinogens and inferred that the complex was that of iron-nitric oxide-thiol protein. Later, Nagata *et al.* (7) carried out similar experiments *in vitro* and obtained a similar electron spin resonance signal. They suggested that the most probable structure of the complex was




Neither group, however, isolated and identified the complex.

In a study of the etiological factors in esophageal cancer, we isolated and identified, by GC[3]-MS, RRME, having the structure




from pickled vegetables of Linxian County, North China, where people suffer from a high incidence of esophageal cancer (9, 11). We have also studied the chemical properties of RRME and found that it reacts readily with secondary amines to form nitrosamines both *in vitro* and *in vivo* (10). At the same time, the biological action of RRME has been studied to show that it is a mutagen (4) and a tumor-promoting agent (1) which can induce forestomach papillary tumors in mice (3). A question has thus arisen as to whether RRME occurring in the pickled vegetables of Linxian is a factor responsible for esophageal cancer in that area. A method for quantitative analysis of RRME in the pickled vegetables has been developed with a GC-MS high-resolution single-ion monitoring technique, and the contents of RRME in pickled vegetables from Linxian and Beijing are compared in this report.

## MATERIALS AND METHODS

### Isolation Procedure

**Drying.** The pickled vegetables[4] were air dried in a ventilating cabinet at room temperature and then dried over silica gel until constant weight (about one-tenth of the undried pickled vegetables) was obtained.

**Extraction.** Dried pickled vegetables (10 g) were placed in a 150-ml Erlenmeyer flask and soaked 3 times, for 4 hr each, with 100-ml portions of dichloromethane. The extracts were combined, transferred to another Erlenmeyer flask, and concentrated to 2 to 3 ml with a gentle stream of nitrogen. All processes were carried out at room temperature and in the dark.

**Column Clean-up.** A glass column (20 x 1.7 cm inside diameter) filled with 50 ml silica gel (100 to 120 mesh) was used for sample clean-up. The silica gel was activated at 110° for 2 hr just before use. The concentrated extract was placed directly on the column, and RRME was eluted with dichloromethane. The first orange-red zone eluted with about 50 ml solvent was collected and concentrated to 1 ml under a gentle stream of nitrogen and then used for GC-MS analysis. The whole procedure was carried out in the dark.

### GC Analysis

A PYE-104 gas chromatograph was used with a 0.5-m × 1.5-mm (inside diameter) glass column filled with 5% OV-17/Chromosorb W (HP) and silanized with dimethyldichlorosilicane (DMCS) (100 to 120 mesh) at 180°. The carrier gas was helium at a flow rate of 20 ml/min.

### MS Analysis

An AEI-MS 50 high-resolution mass spectrometer was used: resolution power, 7000; electron energy, 70 eV; ion source temperature, 150°; separator, silicon membrane at 150°; ion-accelerating voltage, 8 kV.

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[1] Part 1 is the paper of Wang Guang-hui *et al.* (11).

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[3] The abbreviations used are: GC, gas chromatography; MS, high-resolution mass spectrometry; RRME, Roussin red methyl ester.

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[4] Pickled vegetables are made as follows. Assorted vegetables (Chinese cabbages; roots, stems, and leaves of turnips and potherb mustard; etc.) are washed and dipped into boiling water for a few minutes, taken out, let stand until cool, and then tightly packed into a glassed earthen jar with a heavy piece of stone pressing on top for 1 to 2 weeks.

## RESULTS AND DISCUSSION

For quantitative analysis of RRME, it should be considered that some mercaptans, such as cysteine, etc., can react with ferrous salts and nitric oxide in aqueous solutions to form complexes of iron and nitric oxide (6). Since abundant nitrite ions have been found in the pickled vegetables of Linxian in which mercaptans and ferrous salts are also likely to occur, the experimental conditions should be such that the formation of RRME from these precursors is prevented. On the other hand, any iron-thiol protein which is likely to occur in the vegetables might undergo degradation and nitrosation to form RRME; therefore, mild conditions of extraction are required. Another possibility is that some compounds analogous to RRME may also be present in the sample; therefore, care must be taken to avoid possible errors.

**Isolation.** Various isolation methods were tested.

Steam distillation (8) has been used widely for isolation of volatile nitrosamines. The original aim of using this technique in our study was to analyze concurrently both RRME and the nitrosamines. However, serious sublimation was observed on the wall of the condenser, and the recovery of RRME was very low.

Soxhlet extraction of dried pickled vegetables was conducted with dichloromethane in a Soxhlet extractor for 15 hr, and the extract was then cleaned by passage through a silica gel column. The recovery was still rather poor, evidently because the stability of RRME is too low to withstand the refluxing temperature of 39–41° for such a long time and serious decomposition or sublimation occurred.

Ether extraction was carried out at room temperature, and the extract was also cleaned by passage through a column of silica gel. The result was still not satisfactory owing to low separating efficiency of the clean-up process, indicating an improper solvent.

The above results show that satisfactory recovery of RRME without change of chemical composition requires a proper extracting solvent and mild conditions. A satisfactory procedure was thus chosen in which RRME was extracted with dichloromethane, giving a much better separation efficiency in the clean-up process, and the whole process was carried out at room temperature and in the dark. Under these mild conditions, the formation of RRME from precursors originally present in the vegetables should also be prevented. When samples made by adding 0.3 mg of RRME to 100 g of undried pickled vegetables were tested, the recovery of RRME by this procedure was 78 ± 9% (S.D.) (average of 5 measurements).

Table 1 gives a comparison of yields by different isolation methods.

**GC-MS-High-Resolution Single-Ion Monitoring.** The GC-MS single- or multiple-ion monitoring techniques have long been accepted as sensitive and reliable methods for quantitative analysis of organic compounds (5). In our experiment, the technique of high-resolution single-ion monitoring combined with peak matching was used. A continuous bleeding of perfluorokerosene was used for sensitivity calibration as well as accurate mass reference. When the resolution power is 7000, a mass measurement accuracy of 3 ppm can be obtained; thus, this method gives higher reliability than simply using ion-monitoring technique.

With this method, the base peak of RRME, i.e., $m/z$ 295.8549, and a PFK peak, $m/z$ 292.9824, were focused. It was found that all 10 samples of pickled vegetable from Linxian gave $m/z$ 295.8549 ion. The content of RRME was calculated by comparing the intensity of the $m/z$ 295.8549 ion with a calibration curve derived from GC injection of various amounts of synthetic RRME.

The following measurements were also carried out to check the validity of the analytical results: (*a*) the molecular peak of RRME, $m/z$ 325.8529, was also monitored, and the ratio of the peak heights of $m/z$ 325.8529 and 295.8529 of the isolated compound was measured to be 70% which agreed with the corresponding ratio of the synthetic RRME; (*b*) when the isolated compound eluted from the GC column, a low-resolution full mass spectrum ranging from $m/z$ 400 to $m/z$ 20 was obtained, which coincided with that taken from the synthetic RRME; (*c*) the retention time of the isolated compound was found to be the same as that of the synthetic RRME. These results further confirmed that the isolated compound is RRME.

The contents of RRME in pickled vegetables, 10 samples from Linxian and 3 samples from Beijing, are shown in Table 2.

The marked differences between the RRME content in pick-

Table 1

*Comparison of different isolation methods*

Samples of pickled vegetables were taken from different families in Linxian.

| | Content of RRME (mg/kg) | | | |
|---|---|---|---|---|
| | Steam distillation | Soxhlet extraction | Ether extraction | Dichloromethane extraction at room temperature |
| Sample 1 | | | | |
| Experiment 1 | 0.0 | 0.2 | | 0.8 |
| Experiment 2 | 0.0 | 0.2 | | 0.9 |
| Sample 2 | | | | |
| Experiment 1 | 0.2 | 1.4 | | 4.1 |
| Experiment 2 | 0.2 | 1.7 | | 2.9 |
| Sample 3 | | | | |
| Experiment 1 | | | 0.2 | 0.6 |
| Experiment 2 | | | | 0.5 |

Table 2

*Content of RRME in pickled vegetables from Linxian and Beijing*

| | Content of RRME (mg/kg)[a] | | |
|---|---|---|---|
| Pickled vegetables | Experiment 1 | Experiment 2 | Av. |
| From Linxian[b] | | | |
| 1 | 0.8 | 0.9 | 0.8 |
| 2 | 4.1 | 2.9 | 3.5 |
| 3 | 1.0 | 1.3 | 1.2 |
| 4 | 3.9 | 5.1 | 4.5 |
| 5 | 1.8 | 1.7 | 1.8 |
| 6 | 0.3 | 0.3 | 0.3 |
| 7 | 0.2 | 0.3 | 0.2 |
| 8 | 0.5 | 0.3 | 0.4 |
| 9 | 0.1 | 0.1 | 0.1 |
| 10 | 0.6 | 0.5 | 0.6 |
| From Beijing | | | |
| Salted xuelihong[c] | 0.005[d] | | |
| Unsalted xuelihong | 0.005 | | |
| Sour cabbage | 0.005 | | |

[a] Content in undried pickled vegetables.
[b] Samples taken from different families in Linxian.
[c] A species of potherb mustard.
[d] Detection limit of the analytical method.

led vegetables from Linxian County and Beijing might be one of the possible reasons for differences in esophageal cancer incidence between the 2 regions.

**Direct Sample Insertion-High-Resolution Single-Ion Monitoring.** When a sample is analyzed by GC-MS-single-ion monitoring, it must pass through the GC column before entering the mass spectrometer. In these experiments, the GC column was held at a rather high temperature (180°), and the retention time of RRME on the column was about 2 min. It is possible that the sample underwent chemical decomposition and that the RRME detected by mass spectrometry was actually a degradation product of a more complex structure. Therefore, the concentrated dichloromethane extracts of pickled vegetables obtained prior to the column clean-up process were analyzed by direct sample insertion. The sample was placed in the insertion probe without heating and introduced into the ion source. If there were any RRME inherently present in the sample, it would sublime in the vacuum to give ions of $m/z$ 296 and $m/z$ 326 soon. The experiment showed that these ions did appear as expected. Their accurate masses were measured by peak-matching technique and were found to agree, respectively, with the accurate masses of the base peak and molecular peak of RRME within 3 ppm.

## REFERENCES


1. Cheng, S. J., Sala, M., Li, M. H., Courtois, I., and Chouroulinkov, I. Promoting effect of Roussin's red identified in pickled vegetable from Linxian, China. Carcinogenesis (Lond.), *2:* 313–319, 1981.
2. Chiang, R. W., Woolum, J. C., and Commoner, B. Further study on the properties of the rat liver protein involved in a paramagnetic complex in the liver of carcinogen-treated rats. Biochim. Biophys. Acta, 257: 452–460, 1972.
3. Li, M. H., Lu, S. H., Ji, C., Wang, Y. L., Wang, M. Y., Cheng, S. J., and Tian, G. Z. Experimental studies on the carcinogenicity of fungus-contaminated food from Linxian County. *In*: H. V. Gelboin, B. Macmahon, T. Matsushima, T. Sugimura, S. Takayama, and H. Takebe (eds.), Genetic and Environmental Factors in Experimental and Human Cancer, pp. 139–148. Tokyo: Japan Scientific Societies Press, 1980.
4. Lu, S. H., Camus, A. M., Tomatis, L., and Bartsch, H. Mutagenicity of extracts of pickled vegetables collected in Linhsien County, a high-incidence area for esophageal cancer in Northern China. J. Natl. Cancer Inst., *66:* 33–36, 1981.
5. McCamish, M. Quantitative mass spectrometry in pharmacological science. J. Mass Spectrum. Biochem. Med. Environ. Res., *1:* 7–31, 1981.
6. McDonald, C. C., Phillips, W. D., and Mower, H. F. An electron spin resonance study of some complex of iron, nitric oxide, and anionic ligands. J. Am. Chem. Soc., *87:* 3319–3326, 1965.
7. Nagata, C., Ioki, Y., Kodama, M., Tagashira, Y., and Nakadate, M. Free radical induced in rat liver by a chemical carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Ann. N. Y. Acad. Sci., *222:* 1031–1047, 1973.
8. Sen, N. P., and Dalpe, C. A simple thin-layer chromatographic technique for the semi-quantitative determination of volatile nitrosamines in alcoholic beverages. Analyst, 97: 216–220, 1972.
9. Wang, M. Y., Lu, S. H., Ji, C., Wang, Y. L., and Li, M. H. The occurrence of a nitroso compound, Roussin red, and its precursors in pickled vegetables from Linxian County. Chinese J. Oncol., in press, 1983.
10. Wang, M. Y., Li, M. H., Jiang, Y. Z., Sun, Y. H., Li, G. Y., Zhang, W. X., Chai, W. G., and Wang, G. H. Nitrosation of secondary amines with Roussin red methyl ester isolated from Linxian pickles. Chinese J. Oncol., in press, 1983.
11. Wang, G. H., Zhang, W. X., and Chai, W. G. The identification of natural Roussin red methyl ester. Acta Chim. Sinica, *38:* 95–102, 1980.
12. Woolum, J. C., and Commoner, B. Isolation and identification of a paramagnetic complex from the livers of carcinogen-treated rats. Biochim. Biophys. Acta, *201:* 131–140, 1970.

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