

ISOLATION AND IDENTIFICATION BY
MASS SPECTROMETRY OF PHYTOTOXINS IN
COFFEA ARABICA⁽¹⁾

CHANG-HUNG CHOU and GEORGE R. WALLER

*Institute of Botany, Academia Sinica,
Taipei, Taiwan 115, Republic of China*

and

*Department of Biochemistry, Oklahoma State University,
Stillwater, OK 74074, U. S. A.*

(Received December 3, 1979; Accepted January 3, 1980)

Abstract

Eleven phytotoxic substances, isolated from chloroform and ether extracts of aqueous infusions of coffee roots, fallen leaves, and young seedlings, were identified by paper and thin-layer chromatography and mass spectrometry. These are *p*-hydroxybenzoic, vanillic, *p*-coumaric, ferulic, chlorogenic, and caffeic acid, and scopoletin as plant phenolics, and caffeine, theobromine, theophylline, and paraxanthine as purine alkaloids. Caffeine was predominant in all extracts. Mass spectrometry of these eleven compounds showed significant metastable peaks and fragment ions varying with the compounds even though certain ones had the same molecular weight.

Introduction

Coffea arabica, one of the most important beverage plant for humans, is also used for the production of caffeine, mainly from the fruit. The coffee plantations exhibit significant allelopathic phenomenon (Anaya *et al.*, 1978), and eleven allelopathic substances including four alkaloids and seven phenolic compounds were reported in the aqueous extracts of coffee plant parts (Chou and Waller, 1980). They found that plant phenolics and caffeine were abundant in the fallen leaves of old mature plants, whereas less phenolics and more alkaloids were present in the young seedlings. The identification was mainly done by paper and thin layer chromatography and mass spectrometry. Although we have reported these compounds, the mass spectrometric data of the eleven phytotoxins has not fully been described (Spiteller *et al.*, 1961; Chai-

(1) Paper No. 223 of the Scientific Journal Series, Institute of Botany, Academia Sinica, Taiwan, Republic of China, and Journal Article No. 3585 of the Agricultural Experiment Station, Oklahoma State University, Stillwater, Oklahoma, U. S. A..

gneau *et al.*, 1968; Midha *et al.*, 1977). Thus, we present here the detailed information concerning the isolation and identification of compounds and the metastable peaks and the possible fragmentation patterns of these phytotoxins in *C. arabica*.

Material and Methods

Materials

The fallen leaves of 2-year-old coffee plants⁽²⁾ were obtained from the horticultural greenhouse of the Agricultural Experiment Station, Oklahoma State University, U.S.A., and young seedlings about 10 cm in length were collected from the Kew Gardens, London, United Kingdom in 1977. The plant materials were dried at room temperature, ground, and stored in a desiccator before using. Authentic known compounds were purchased from Sigma Company, U.S.A., and Dr. Bender—Dr. Hobein, A.-G., Switzerland. Solvents were obtained from Burdick & Jackson, U.S.A.

Isolation of phytotoxic substances

Four grams of each plant sample and 96 ml of distilled water were shaken together for 2 hr. The aqueous extracts were filtered through Whatman 41 paper. The residue was subjected to reextraction with distilled water twice. All filtrates were combined and concentrated in vacuo below 45°C; the concentrates were extracted with ethyl ether three times (100 ml each time). The ether extracts were combined, evaporated in a stream of nitrogen, redissolved with 1 ml of methanol-chloroform (1: 1, v/v), and stored in vials. The aqueous fraction was further treated by adding 50 ml 0.0125 N H₂SO₄, refluxing for 30 min., subsequently adding 5 g of MgO, and refluxing for about 30 min more. The mixture was filtered and the filtrate was concentrated to near dryness and extracted with chloroform three times. The chloroform extract was evaporated in a nitrogen stream and redissolved with 2 ml chloroform.

Purification and identification of phytotoxins

To purify and identify the phytotoxins present in *C. arabica*, paper and thin-layer chromatography (PC and TLC) were employed. In PC, a paper strip designed by Wang *et al.* (1967) of Whatman 3 MM chromatographic paper was spotted, and developed with 2% acetic acid by descending chromatography. After developing, the paper was dried and examined under ,UV (2357 nm) and ,UV (3660 nm) light viewers to locate the spots. One of the chromatograms

(2) The *C. arabica* plants were supplied by the United States Department of Agriculture Beltsville, Md., which obtained them from Ethiopia via Oreiras, Portugal.

servicing as a reference was sprayed with diazotized *p*-nitroaniline (Chou and Young, 1975) followed by 20% Na₂CO₃. The known authentic compounds were also co-chromatographed simultaneously with unknown samples. The compounds on the chromatogram were eluted with methanol. The eluate was used either for TLC or mass spectrometric analysis (MS).

For TLC, on an analytical plate (0.2 mm thickness, Silica Gel 60F-254, E. Merck, Germany) 10 μ l of extract or solution of known compounds was spotted and the plate developed with two solvent systems, chloroform—ethanol (90:10, v/v), and acetic acid—chloroform (10:90, v/v) (Harborne, 1973), in either one- or two- dimensional chromatography. On a preparative TLC plate (2 mm thickness, Silica GF, Analtech, Inc., U.S.A.) 300 μ l of extract was spotted and development performed with the same solvents. The spots visualized under the UV light were eluted with methanol. The purification process was done several times, and the final purified compound(s) was subjected to MS analysis.

Mass spectrometric analysis

In MS analysis, enough eluate was placed in a vial for use in a direct probe by the method described by Waller and Lawrence (1978) and analyzed with a prototype LKB 9000 GC-MC (Waller, 1968, 1972; Waller and Dermer, 1979). The known compounds were run by the same techniques. High resolution mass spectra were obtained on a DuPont/CEC 21/110D by Catherine B. Costello at the MIT Biotechnology Research Resource for Mass Spectrometry.

Results and Discussion

From the R_f values in paper and thin-layer chromatography (PC and TLC) in different solvent systems, response to UV light, and color reactions produced by spray reagents, phytotoxins present in *C. arabica* were identified (Chou and Waller, 1980). The PC and TLC properties of each isolated compound agree with those of a known synthetic compound. The phytotoxins are caffeic, chlorogenic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, and vanillic acids, and scopoletin, theobromine, theophylline, paraxanthine, and caffeine. Among these compounds, caffeine is the most abundant one (Fig. 1). The caffeine content was significantly higher in young seedlings than in fallen leaves and roots (Fig. 1). Theobromine, theophylline, and paraxanthine were found in the coffee seedlings but paraxanthine was absent in the fallen leaves. On the other hand, more phenolics were present in the fallen leaves than in the young seedlings.

Each isolated phytotoxic compound was subjected to mass spectrometric

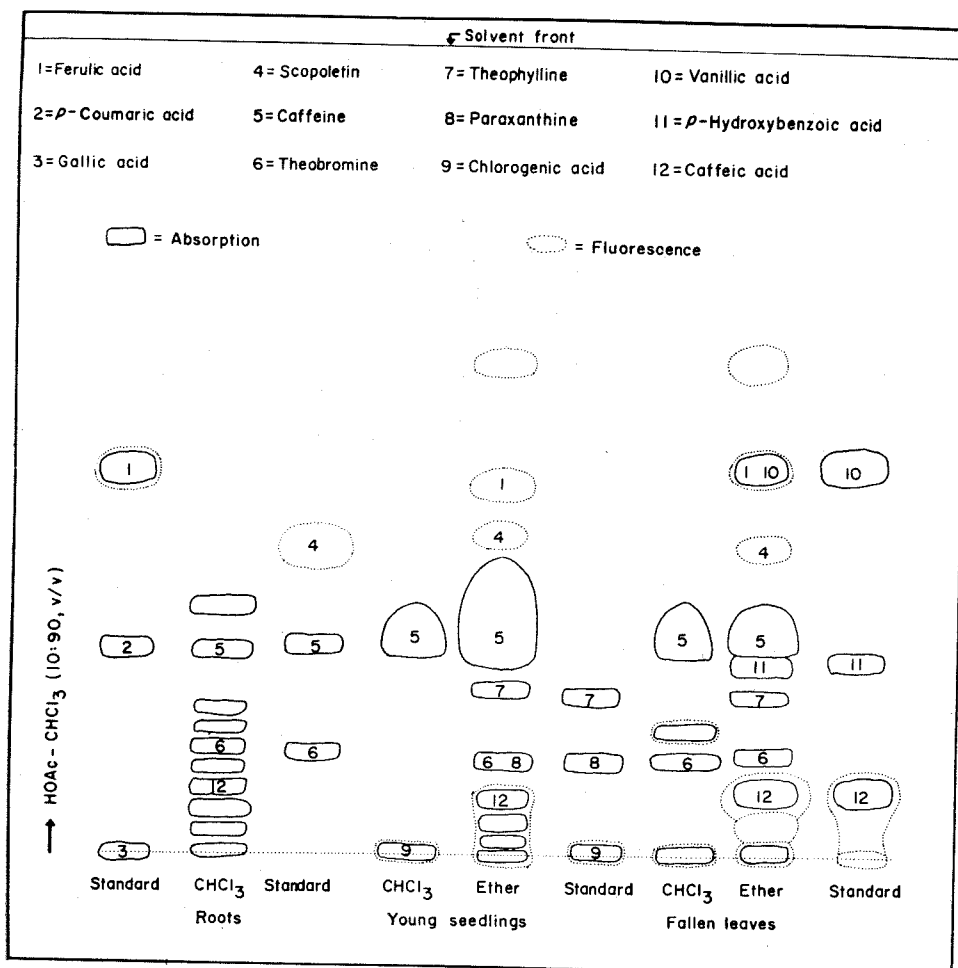


Fig. 1. Tracing of a thin-layer chromatogram of standard compounds and the chloroform and ether fractions of aqueous extracts of coffee plant parts. The plate gave an absorbing spot, except scopoletin, under λ UV light and a fluorescent spot under λ UV light.

(MS) analysis, and each spectrum was compared with that of known standards and a data bank-library through the Finningan GC-MS-Computer Search System (courtesy of Continental Oil Company, Ponca City, Oklahoma, U.S.A.). The spectra of the isolated compounds agree with those of standards. The reference data bank lacks a record for scopoletin and such is reported (Fig.2).

Metastable peaks (Waller, 1972) occurred in the spectra of the phytotoxic phenolic compounds (Table 1). For example, chlorogenic acid exhibited one metastable peak, while ferulic acid showed seven. The probable neutral products varied with compounds; they include COOH , H_2O , OH , CO , CH_3 , C_2H_2 , C_2H_3 , and O . Scopoletin shows successive losses (Scheme I) of CH_3 and CO

forming m/z 177 and 164 respectively and each of the latter ions undergoes additional fragmentation giving losses of CO with the resulting ion being formed at m/z 149. Loss of CH_3 group from m/z 164 occurs to give another ion at m/z 149. The loss of the ions (CO + CO) from m/z 177, and loss of CO from each of the fragment ions of m/z 149, (although it was from a different portion of the fragment ion at m/z 149) give rise to m/z 121 (Scheme 1).

Table 1. *Metastable peaks in mass spectra of C. arabica phenolics*

| Compound | Apparent mass (m^*) ⁽¹⁾ | Transition denoted | | Probable neutral product |
|-------------------------------|---|--------------------|----------|-------------------------------|
| | | m_1 | m_2 | |
| Caffeic acid | 101.3 | 180 | → 135+45 | COOH |
| | 128.9 | 163 | → 145+18 | H ₂ O |
| | 147.6 | 180 | → 163+17 | OH |
| Chlorogenic acid | 128.9 | 163 | → 145+18 | H ₂ O |
| Ferulic acid | 56.5 | 105 | → 77+28 | CO |
| | 73.0 | 151 | → 105+46 | CO+H ₂ O |
| | 81.7 | 135 | → 105+30 | CH ₂ O |
| | 101.8 | 179 | → 135+44 | CO ₂ |
| | 127.3 | 179 | → 151+28 | CO |
| | 144.8 | 179 | → 161+18 | H ₂ O |
| <i>p</i> -Coumaric acid | 46.4 | 91 | → 65+26 | C ₂ H ₂ |
| | 70.1 | 118 | → 91+27 | C ₂ H ₃ |
| | 95.4 | 146 | → 118+28 | CO |
| | 129.9 | 164 | → 146+18 | H ₂ O |
| Vanillic acid | 74.6 | 126 | → 97+29 | CHO |
| | 103.8 | 153 | → 126+27 | C ₂ H ₃ |
| | 135.7 | 168 | → 151+17 | OH |
| | 139.2 | 168 | → 153+15 | CH ₃ |
| <i>p</i> -Hydroxybenzoic acid | 71.5 | 121 | → 93+28 | CO |
| | 106.1 | 138 | → 121+17 | OH |
| Scopoletin | 82.7 | 177 | → 121+56 | CO+CO |
| | 98.3 | 149 | → 121+28 | CO |
| | 125.4 | 177 | → 149+28 | CO |
| | 135.4 | 164 | → 149+15 | CH ₃ |
| | 140.1 | 192 | → 164+28 | CO |
| | 163.0 | 192 | → 177+15 | CH ₃ |

⁽¹⁾ $(m)^* = (m_2)^2 / (m_1)$

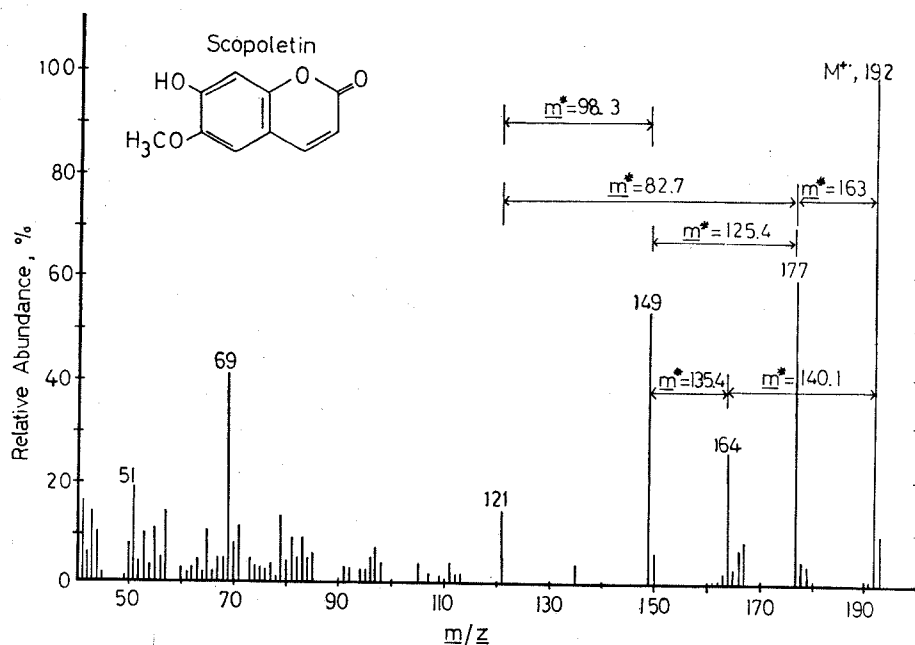


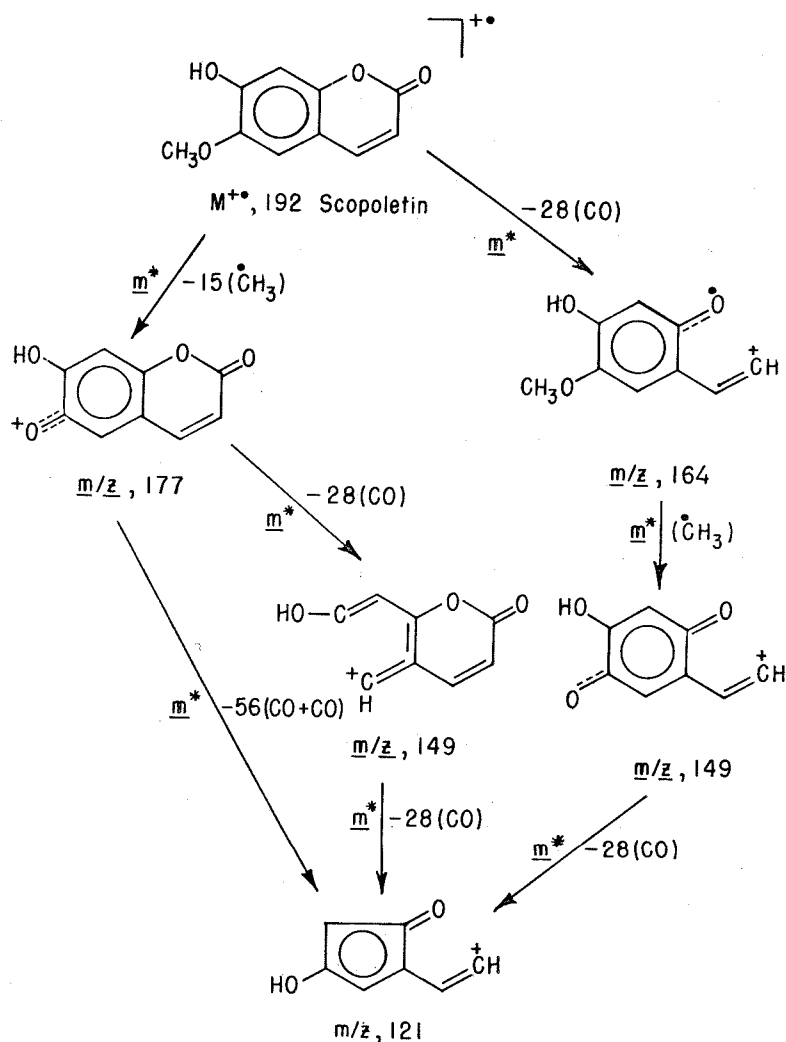
Fig. 2. Mass spectrum of scopoletin. LKB-9000 prototype, 70 eV, 250°C ion source, 100°C inlet, direct inlet.

Table 2. Metastable peaks in mass spectra of *C. arabica* alkaloids

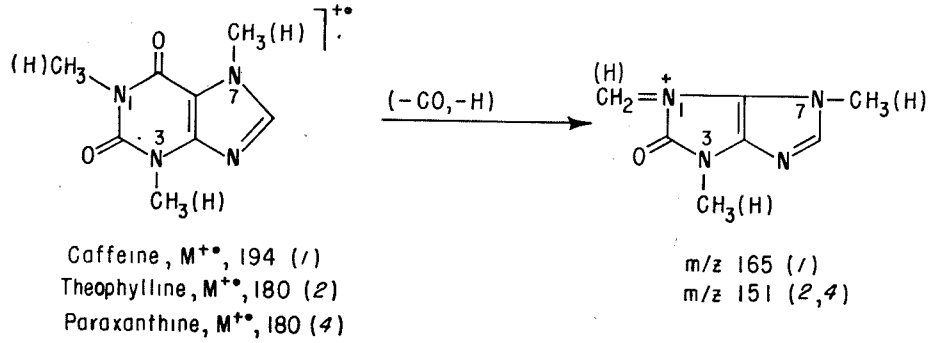
| Compound | Apparent mass (m^*) ⁽¹⁾ | Transition denoted | | Probable neutral product(s) |
|--------------|---|--------------------|----------|--------------------------------|
| | | m_1 | m_2 | |
| Caffeine | 61.2** | 194 | → 109+85 | CH ₃ CNOCO |
| | 88.0** | 135 | → 109+26 | C ₂ H ₂ |
| | 97.3** | 148 | → 120+28 | CO |
| | 106.7*** | 135 | → 120+15 | CH ₃ |
| | 135.3** | 194 | → 162+32 | CH ₃ OH |
| Theobromine | 61.7** | 109 | → 82+27 | HCN |
| | 86.7** | 137 | → 109+28 | CO |
| | 104.3*** | 180 | → 137+43 | CH ₃ +CO |
| Theophylline | 48.7* | 95 | → 68+27 | HCN |
| | 73.4** | 123 | → 95+28 | CO |
| | 84.1** | 180 | → 123+57 | CH ₃ -N=C=O |
| Paraxanthine | 48.7* | 95 | → 68+27 | HCN |
| | 73.4*** | 123 | → 95+28 | CO |
| | 84.1*** | 180 | → 123+57 | CH ₃ -N=C=O |
| | 126.7* | 180 | → 151+29 | CHO |

⁽¹⁾ $m^* = (m_2)^2 / (m_1)$; *, **, *** = weak, medium, and strong, respectively.

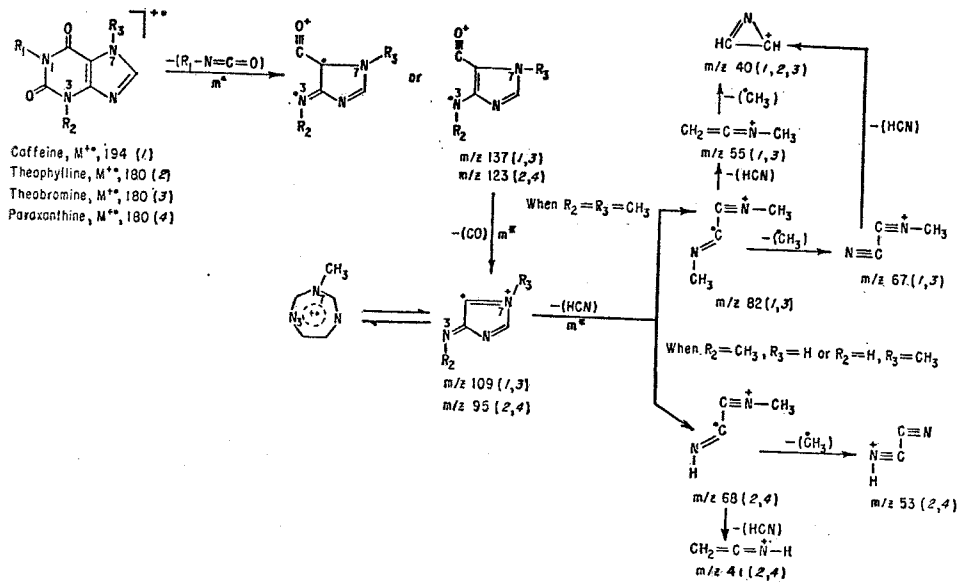
The metastable peaks of four purine alkaloids caffeine, theobromine, theophylline, and paraxanthine are given in Table 2. All four compounds show the molecular ion as the base peak, reflecting the stability of aromatic heterocyclic ring system toward fragmentation upon impact (Budzikiewicz *et al.*, 1964; Hignite, 1972; Midha *et al.*, 1977). We proposed two possible fragmentation pathways for the decomposition of purine alkaloids (Scheme 2, and Scheme 3). Except caffeine, the fragmentation patterns of the remaining compounds were almost the same as those described by Midha *et al.* (1977). The decomposition pathways involving metastable peaks serve to confirm the pathway for a given molecule and coincidence of the data for the known and the



Scheme 1. Partial fragmentation pattern of scopoletin.



Scheme 2. Partial fragmentation pattern of caffeine, theophylline and paraxanthine.



Scheme 3. Possible fragmentation patterns of caffeine theophylline, theobromine and paraxanthine.

unknown, permit the conclusive identification of plant phenolics and the purine alkaloids.

From the ecological point, it is reasonable to extract phytotoxic compounds initially with water because most such compounds are water soluble. However, some phytotoxins may remain in the plant material after aqueous extraction, and any such compound has been missed in this study. In addition, several unknown compounds found by PC and TLC (Fig. 1) have not been identified even by MS analysis. Since the MS analysis was not conclusive the compounds will be derivatized and reported later.

Acknowledgement

We are grateful to Drs. Otis C. Dermer, Tom J. Mabry, and A. Ulubelen for their critical comments on the manuscript, and to Mrs. Cheryl Cumberland for technical assistance. The senior author thanks the National Science Council of Republic of China of supporting his Sabbatical Leave of Absence (1978-1979) from Academia Sinica.

Literature Cited

- Anaya, A.L., S. del Amo, G. Roy. Octla and L.M. Ortiz. 1968. Allelopathic potential of a coffee plantation. p. 8 (Abstract), 2nd Int. Cong. Ecol. Jerusalem, Israel.
- Budzikiewicz, H., C. Djerassi and D.H. Williams. 1964. Structure elucidation of natural products by mass spectrometry. p. 233. Volume 1, Alkaloids. Holden-Day, Inc., San Francisco, London, Amsterdam.
- Chaigneau, de MM.M., G. Valdener et M.J. Seyden-Penne and M.R. Fabre. 1965. Étude du spectre de masse de la thiotheophylline. C.R. Acad. Sc. Paris, **260**: 3965-3968.
- Chou, C.H. and G.R. Waller. 1980. Possible allelopathic constituents of *Coffea arabica*. J. Chem. Ecol. **6**: (in press).
- Chou, C.H. and C.C. Young. 1975. Phytotoxic substances in twelve subtropical grasses. J. Chem. Ecol. **1**: 183-193.
- Harborne, J.B. 1973. Phytochemical Methods. Chapman and Hall, London, pp. 33-38.
- Hignite, C. 1972. Nucleic acids and derivatives. p. 387. In G.R. Waller (ed.), Biochemical Applications of Mass Spectrometry. Wiley-Interscience, N.Y.
- Midha, K.K., S. Sved, R.D. Hossie and I.J. McGilveray. 1977. High performance liquid chromatographic and mass spectrometric identification of dimethyl xanthine metabolites of caffeine in human plasma. Biomed. Mass **9**: 172-177.
- Spiteller, G. and M. Spiteller—Friedmann. 1962. Vergleichende massenspektrometrische Untersuchung einiger Purinderivate. Mh. Chem. Bd. **93**: 632-638.
- Waller, G.R. 1968. Description of the Oklahoma State University Combination mass spectrometer-gas chromatograph. Proc. Okla. Acad. Sci. **47**: 271-292.
- Waller, G.R. 1972. Biochemical Applications of Mass Spectrometry. Wiley-Interscience, N.Y. 872 p.
- Waller, G.R. and O.C. Dermer. 1979. Biochemical Applications of Mass Spectrometry. First Supplementary volume, Wiley-Interscience, N.Y. (in press).
- Waller, G.R. and R.H. Lawrence. 1978. Isolation and determination of structures of some new diterpenoid alkaloids in *Delphinium ajacis* by mass spectrometry. pp. 429-439. In A. Frigerio (ed.) Recent Developments in Mass Spectrometry in Biochemistry and Medicine. vol. 1, Plenum Publishing Corporation, N.Y.
- Wang, T.S.C., S.Y. Cheng and H. Tung. 1967. Extraction and analysis of soil organic acids. Soil Sci. **103**: 360-366.

分離及以質譜儀鑑定咖啡植物中之植物毒性物質

周 昌 弘 GEORGE R. WALLER

中央研究院植物研究所 美國奧克拉荷馬州立大學生化學系

咖啡植物 (*Coffea arabica*) 的落葉，根及幼苗的氯仿及水溶等萃取液經紙色層，薄色層及質譜儀之分析鑑定得十一種植物毒物質。其中七種為酚酸類化合物：*p*-hydroxybenzoic, vanillic, *p*-coumaric, ferulic, chlorogenic, caffeic acid 及 scopoletin；另四種為植物鹼化合物：caffeine, theobromine, theophylline 及 paraxanthine。在上述兩萃取液中 caffeine 的含量最高。以質譜儀分析上述化合物得知其準安定離子 (metastable ion) 及分裂離子 (fragmentation ion) 因化合物而異。