Essential Oils and Their Constituents XX

Detection and Estimation of Menthofuran in *Mentha arvensis* and Other Mint Species by Coupled Gas-Liquid–Thin-Layer Chromatography

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The occurrence of menthofuran in *Mentha arvensis* is reported for the first time. From 0.01 to 0.04 per cent of the heterocycle was determined by means of coupled gas-liquid–thin-layer chromatography in genuine essential oils obtained from plants grown in Argentina, Brazil, Formosa, India, Japan, and South Africa. Its presence at such low concentrations could not be detected by conventional chemical tests, *e.g.*, Flückiger's reaction, or by modern instrumental techniques, including ultraviolet and infrared spectrophotometry applied to analytical specimens directly. The classical distinction between *Mentha piperita* (peppermint) and *M. arvensis* (mint), ostensibly based on the presence or absence of menthofuran, is therefore no longer justifiable. Other mint species, including spearmints (*Mentha cardiaca* Gerard ex Baker cultivar Scotch or Highland spearmint, *Mentha spicata* L. cultivar common or native American spearmint, and *Mentha viridis*), pennyroyal (*Mentha pulegium* L. and *Hedeoma pulegioides* (L.) Pers.), bergamot mint (*Mentha citrata* Ehrh.), *Mentha sylvestris* L., and *Mentha rotundifolia*, contained trace amounts of menthofuran also. The experimental data suggest a reappraisal of presently acknowledged concepts regarding both biogenesis and chemotaxonomy of the genus *Mentha*.

ALMOST ONE-HUNDRED years ago Flückiger observed that peppermint oil gave a characteristic color reaction when heated in glacial acetic–nitric acid solution (1). In 1890, Polenske suggested that the phenomenon was due to the presence of a volatile, nonnitrogenous constituent in the essential oil (2). Thirty years later the compound, menthofuran, was isolated by Carles as an "ether-oxide" from the flower oil of Italian peppermint (3). Its constitution was established soon afterwards by Wienhaus and Dewein (4). In 1936, Treibs synthesized the heterocycle and demonstrated that its properties were identical with those of the natural isolate (5).

As a criterion of identity for *M. piperita*, Flückiger's test came to be applied widely and with only slight modifications. It is generally carried out as follows. Three drops of the essential oil are mixed in a dry test tube with 5 ml. of a solution of 1 vol. of nitric acid in 300 vol. of glacial acetic acid, and the tube is placed in a beaker of boiling water. Within 5 minutes the liquid develops a blue color, which on continued heating deepens and shows a copper fluorescence, then fades, leaving a golden yellow solution (6). With *M. arvensis*, this sequence of color changes is not observed, the test solution attaining merely a light yellowish tinge which undergoes no marked change during the 5-minute heating period.

Offering a valuable criterion for distinguishing readily two closely allied and commercially most important products of the genus *Mentha*, the reaction was soon considered and still is an official specification (7, 8). As a spot test requiring but 1 drop of material, it was later utilized by Zolotovich for the examination of *M. arvensis* as well as *M. piperita* oils and many of their hybrids (9).

Studies carried out recently in our laboratory on products of different geographical origins confirmed the specificity generally ascribed to the reaction (10). Yet, not always were experimental data entirely satisfactory, and the investigation was continued. It is the purpose of this paper to report the results that we obtained using coupled gas-liquid–thin-layer chromatography as the method of analysis.

EXPERIMENTAL

Apparatus and analytical procedures were previously described (11).

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Materials

Essential Oils.—Oil of mint (*M. arvensis*) was provided through the courtesy of Dr. Juan-Carlos Tuja, Cultivos Industriales, S.A., Buenos Aires, Argentina, Drs. M. T. Magalhães and O. R. Gottlieb, Instituto de Química Agrícola, Rio de Janeiro, Brazil; Dr. T. S. Chou, Bureau of Commodity Inspection and Quarantine, Republic of China, Taipei, Taiwan; Dr. I. C. Chopra, Regional Research Laboratory, Jammu-Tawi, Kashmir State, India;

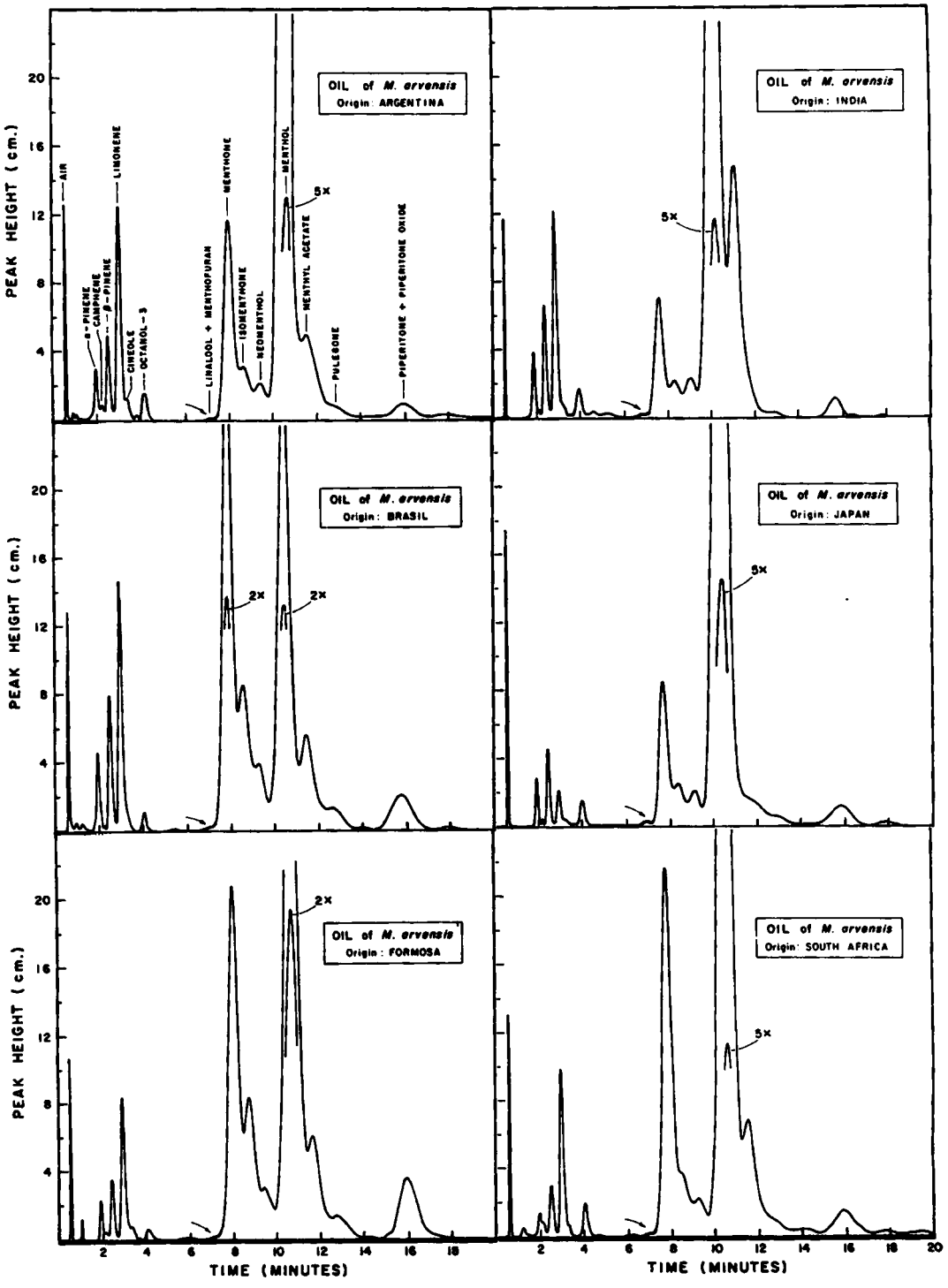

Fig. 1.—Gas chromatograms of *M. arvensis* oils. Analytical peaks reproduced in heavy lines. Column—SAIB (20%) on Chromosorb W (acid-washed), 60–80 mesh; temperature, 170° C.; helium, 75 ml./minute; sample volumes, 2 μl.

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Oil of spearmint (*M. cardiaca, M. spicata, M. viridis*) was supplied through the courtesy of Dr. F. J. Cramer, A. M. Todd Co., Kalamazoo, Mich., and Dr. C. Scholtens, N. V. Chemische Fabriek "Flebo," Hoogezand, The Netherlands.

Oil of pennyroyal (*M. pulegium* L. and *Hedeoma pulegioides* (L.) Pers.) was provided through the courtesy of Dr. E. Guenther, Fritzsche Brothers, Inc., New York, N. Y.; Dr. F. J. Cramer, A. M. Todd Co., Kalamazoo, Mich.; Dr. J. M. Quigg, Compagnie Parento, Ltd., Toronto, Ontario, Canada; Destilaciones Bordas Chinchurreta, S.A., Seville, Spain.

Oil of bergamot mint (*M. citrata* Ehrh.) and oil of *M. sylvestris* L. was supplied through the courtesy of Dr. F. J. Cramer, A. M. Todd Co., Kalamazoo, Mich.

Oil of *M. rotundifolia* was provided through the courtesy of Dr. Sumio Shimizu, Laboratory of Agricultural Chemistry, Shinshu University, Ina-Nagano Ken, Japan.

Essential Oil Constituents.—Menthofuran (ex *M. piperita*, freshly distilled for use as reference standard); $\epsilon^{EtOH}_{max.\ 219\ m\mu}$ = 6,750. *d*-Pulegone (ex *M. pulegium* L.) was provided through the courtesy of Dr. F. J. Cramer, A. M. Todd Co., Kalamazoo, Mich.

Isolation of Menthofuran Fraction from Two Grams of Brazilian Oil of *M. arvensis*

Sample aliquots of 50 μl. each were subjected to gas chromatography (column: SAIB (20%) on Chromosorb W; temperature, 170° C.; helium: 75 ml./minute) (12) and effluents emerging just prior to menthone (retention time ~6.5 to 7.5 minutes) trapped in carbon tetrachloride. Following evaporation of solvent, the residue obtained was examined by infrared and ultraviolet spectroscopy, thin-layer chromatography, and Flückiger's classical color test.

Coupled Gas-Liquid–Thin-Layer Chromatograms of Essential Oils

Samples ranging from 25–100 μl. were injected into the column and fractions emerging within 6.5 to 7.5 minutes (see Fig. 1) deposited directly on chromatoplates mounted at the vapor exit as previously reported (11). *n*-Hexane was applied for the development of condensates thus spotted, and a 5% solution of vanillin in concentrated sulfuric acid served as spray reagent.

Identification and Estimation of Menthofuran in Analytical Specimens

To 10-Gm. samples of an authenticated Japanese oil of *M. arvensis* (Flückiger test negative) were added accurately weighed amounts of menthofuran and preparations containing from 0.10 to 1.00% of the heterocycle made up as reference standards. They served as controls for compound identification and the appraisal of product recovery. Following deposition of 0.5 to 1-μl. aliquots of these mixtures on chromatoplates, development in *n*-hexane for about 45 minutes, and treatment with the 5% vanillin-sulfuric acid reagent, characteristic deep orange-red spots were readily observed. On prolonged standing, they developed a bluish-purple hue.

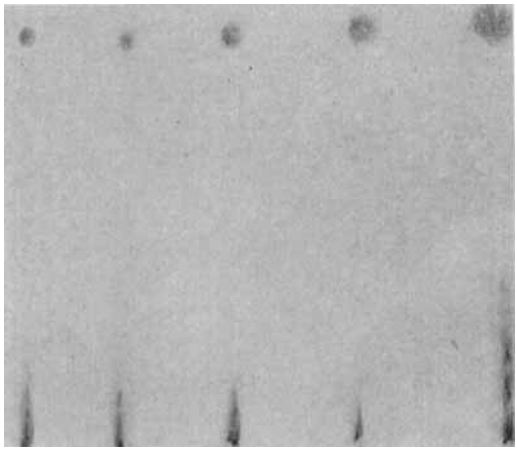
RESULTS AND DISCUSSION

Characterization of Menthofuran Fraction Obtained from Brazilian *M. arvensis*

Infrared analysis of the isolate, prepared in accordance with the procedure described and spread as a thin film between two salt plates, showed weak yet distinct bands characteristic of menthofuran at 1680, 1640, 1565, and 735 cm.$^{-1}$ Marked absorptions throughout the 1200 and 920 cm.$^{-1}$ regions indicated admixture with menthone and linalool, respectively. A Perkin-Elmer double beam, model 221 spectrophotometer equipped with prism grating interchange and automatic gain control was used to make the measurements. It was operated at fivefold scale expansion and flushed with dry nitrogen during the experiment. Although the analytical specimen was of heterogeneous composition, purification by rechromatography was not attempted for fear of recovering insufficient material to carry out further tests.

The crude isolate was therefore subjected to direct ultraviolet analysis and its U.V. characteristics compared with those of the essential oil from which it had been obtained. Taken up in 25 ml. of ethyl alcohol it exhibited peak absorbance (ϵ = 2.1) at 220 mμ, indicative of the presence of approximately 0.03% of menthofuran in the essential oil.

Similar examination of a number of *M. arvensis* oils showed invariably the occurrence of strong maxima or shoulders throughout the 220–240 mμ region; the specimen which had served as starting material for preparation of the menthofuran concentrate displayed a maximum at 231 mμ ($E^{1\%}_{1\ cm.}$ = 43.9). Hence the presence of trace amounts of



1 *M. arvensis* | *2* *M. spicata* | *3* *M. cardiaca* | *4* Menthofuran | *5* Isolate from *M. arvensis*

Fig. 2.—CGT-Chromatograms of analytical peaks from *1, M. arvensis; 2, M. spicata; 3, M. cardiaca;* and TL-Chromatograms of *4*, menthofuran standard, and *5*, menthofuran isolate from *M. arvensis* as described under *Experimental.*

menthofuran in these oils escaped recognition by direct ultraviolet analysis. Thus, the addition of as much as 1% of the heterocycle to genuine preparations could not be detected with any degree of reliability. It is because of such relatively intense absorption by nonmenthofuran constituents throughout the analytical wavelength range that *M. piperita* oils fail, likewise, to submit to menthofuran determinations by direct ultraviolet spectrophotometry.

Following recovery of the concentrate from its ethanolic solution, a 1-μl. aliquot was examined along with a 1-μl. aliquot of reference standard (0.1% menthofuran) by thin-layer chromatography using *n*-hexane as developing solvent. Deep orange-red colorations of identical R_f values were observed following treatment of the chromatoplate with the spray reagent (see Fig. 2).

The remainder of the analytical sample was used for observing Flückiger's reaction. The characteristic sequence of colorations was seen clearly and a strong positive test obtained within 1 minute. Thus, each of the analyses had supplied ample evidence for the presence of menthofuran in *M. arvensis*, and its occurrence in this species can now be considered ascertained beyond doubt.

The most convenient and perhaps also the most convincing of the criteria of identity established were those assembled by thin-layer chromatography carried out under the experimental conditions specified. Of a great many terpene compounds examined similarly in our laboratory, none showed the migration characteristics plus color response typical of menthofuran. Pertinent chemical and chemotaxonomic applications of the technique were reported (13), and further data illustrative of its scope for the analysis of essential oils and their constituents are to be published elsewhere.

TABLE I.—TLC AND CGTC OF ESSENTIAL OILS OF THE GENUS *Mentha*

Essential Oil	TLC Sample Vol., 2 μl.	CGTC Sample Vol., 25–100 μl.	Menthofuran, %
Mint			
M. arvensis L.			
Argentina	−	+	0.01
Brazil APPA-MA-1	−	+	0.02
APPA-MA-4	−	+	0.01
Formosa	−	+	0.03
India	−	+	0.04
Specimen obtained from Japanese mint plants raised and processed in State of Kashmir			
Japan "Sanbi"	−	+	0.04
"Hakubi"	−	+	0.02
South Africa	−	+	0.03
Strain of imported Japanese mint			
Spearmint			
M. cardiaca Gerard ex Baker cultivar Scotch or Highland	−	+	0.02
M. spicata cultivar common or native American	−	+	0.01
M. viridis	−	+	0.02
Pennyroyal[a]			
M. pulegium L.			
Spanish	+	+	0.30
Moroccan	+	+	0.25
Hedeoma pulegioides (L.) Pers.	+	+	0.05
Oregon race	−	+	0.02
M. sylvestris L.[a]	−	+	0.05
M. citrata Ehrh.[a]	+	+	0.07
M. rotundifolia[a]			
French strain, Main constituent neo-neo-isopulegol	+	+	0.06
Italian strain, Main constituent piperitenone oxide	−	+	0.02

[a] Specimens stored prior to analysis under nitrogen and at 5°C. for periods up to 3 years. Menthofuran contents of freshly distilled oils probably higher than reported.

Coupled Gas-Liquid–Thin-Layer Chromatograms

The gas chromatograms of six *M. arvensis* oils of different geographical provenance are shown in Fig. 1.

Qualitatively their profiles are similar. Yet quantitative correlations of peaks observed for biochemically related constituents assures classification of these oils according to geographical origin (10).

In each of the chromatograms a minor peak or small shoulder precedes the emergence of menthone. Shown in heavy lines it illustrates the effluent examined by coupled gas-liquid–thin-layer chromatography as described under *Experimental*. Codeposition of linalool and menthone as well as other constituents emerging during collection of the effluent does not interfere with the analyses because of the selectivity of the solvent system and spray reagent applied. In each instance color intensities observed were compared with those produced by accurately measured aliquots of menthofuran (0.1%)–*M. arvensis* calibration standards examined under the same experimental conditions. The presence of 0.01 to 0.04% of menthofuran as a genuine constituent of *M. arvensis* was thus established (see Table I).

Correlation of these results with those reported in previous investigations (10, 11, 14, 15) illustrates the occurrence of a given constituent at vastly different concentrations in various species belonging to a given genus. Such findings will make imperative revisions of our present concepts concerning the biogenesis of essential oils and their constituents and aid in the development of more reliable methods of plant classifications by physicochemical techniques. Because of its insensitivity, Flückiger's test, as this study has shown, may still be considered of distinct *practical* value to the industry. Yet its fundamental *biogenetic* significance and *chemotaxonomic* importance, as implied in the literature throughout the years, no longer exist.

One of the *M. arvensis* oils assayed, a commercial sample obtained from England, gave a positive menthofuran test (0.14%) when 2-μl. aliquots were spotted for direct thin-layer chromatography. Since adulteration of oils of *M. arvensis* with oils of *M. piperita* would be unprofitable, the observation is probably indicative of genuine compositional variations to be encountered. Quite likely, menthofuran concentrations up to 0.2% are still true criteria of *M. arvensis* authenticity.

For a time it was thought that contamination of *M. arvensis* with wild growing plants might account for the experimental observations. Accordingly, a Brazilian oil from such a species,[1] grown experimentally in the region of Colorado (Paraná State), was subjected to similar analyses. It contained 0.12% of menthofuran. (Pulegone content ~50%.) Comparable data were obtained for an American *M. arvensis* oil[2] produced from plants grown experimentally in Wisconsin.

It would thus appear that the presence of menthofuran in *M. arvensis* cannot be ascribed to contamination with wild growing mints. Since, furthermore, and for the purpose of clarifying this point, essential oils of different geographical origins were analyzed—some of them produced from authenticated strains cultivated under carefully controlled conditions—the possibility of the presence of menthofuran *via* admixture should also be ruled out. Contamination with peppermint can likewise be discounted, for *M. piperita* is not grown in Brazil, Formosa, and Japan. Only experimental plantations exist at this time in Argentina and India. Finally, and equally important is the fact that commercially exploited *M. arvensis* fields in the various producing countries are carefully supervised at all times to insure that only essential oils of highest quality and uniformity are reaching the world's markets. Menthofuran is, evidently, a genuine constituent of the species itself.

Sensitivity of Analytical Techniques

Examination of samples by the glacial acetic–nitric acid reaction proceeded as expected for *M. arvensis* oils. Faintly yellowish solutions formed without intermediate colorations being observed. Similar analyses of menthofuran–*M. arvensis* calibration standards proved that positive tests were obtained only at menthofuran concentrations equal to or greater than 0.4% by weight. Thin-layer chromatography yielded distinctly positive results when applied to 1-μl. aliquots of menthofuran–*M. arvensis* reference standards containing as little as 0.1% of menthofuran by weight. *M. piperita* oils examined similarly gave strongly positive tests with less than 0.5 μl. sample aliquots necessary for analysis. Having at our disposal reliable calibration standards, we screened conveniently menthofuran

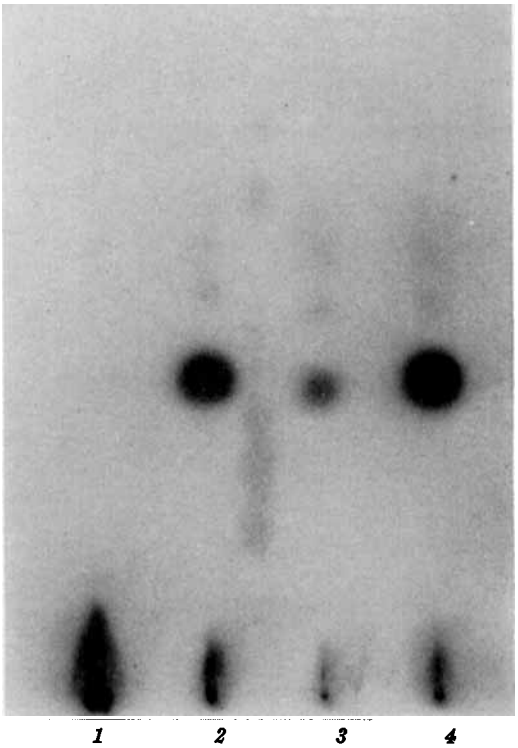


Fig. 3.—Thin-layer chromatograms of *1*, *M. arvensis* (presence of menthofuran not detected); *2*, *M. piperita* (Italo-Mitcham)—menthofuran 6%; *3*, *M. piperita* (English-Mitcham)—menthofuran 2.5%; and *4*, *M. piperita* (U.S.A.-Yakima)—menthofuran 9%. Sample volume—0.5 μl.

[1] Courtesy of Dr. M. T. Magalhães, Instituto de Química Agrícola, Rio de Janeiro, Brazil.
[2] Courtesy of Dr. F. J. Cramer, A. M. Todd Co., Kalamazoo, Mich.

TABLE II.—SENSITIVITY OF VARIOUS TECHNIQUES

Method of Analysis	Limit of Sensitivity
Ultraviolet spectroscopy	>1%
Flückiger reaction	0.4%[a]
Differential infrared spectroscopy	0.25%
Thin-layer chromatography	0.05% (0.5 mcg./μl.)
CGTC	0.01%[b]

[a] Sensitivity in solution slightly better than that reported by Zolotovich (0.5%) for spot test carried out on filter paper with but 1 drop of oil and reagent, respectively (9). [b] Subject to further refinement through increase of sample volumes used for analysis. Such refinement does not apply to thin-layer chromatography which as a rule processes larger complex samples less efficiently than smaller ones. Thus, none of the *M. arvensis* oils listed in Table I gave positive menthofuran tests when 10-μl. aliquots were spotted for analysis. The feature is a decided advantage of CGTC over TLC (see Table I) and outweighs the disadvantage of material losses which might occur during gas chromatography.

contents of peppermint oils available in our collection, as shown in Fig. 3.

An infrared method for the estimation of menthofuran in *M. piperita* oils was reported by Naves. Based on absorption intensities observed at 735 cm.$^{-1}$ it permitted detection of 1% of the heterocycle in genuine products. *M. arvensis* oils examined comparably failed to show the analytical band (16). We applied infrared spectroscopy likewise during the course of this study and confirmed Naves' results. None of the essential oils displayed infrared absorptions suggesting the presence of menthofuran. Only when using differential techniques (17, 18) and operating the instrument at fivefold scale expansion was it possible to detect the addition of 0.25% of menthofuran to authenticated preparations. With menthofuran concentrations in *M. arvensis* below this level direct infrared procedures would presently appear to be inadequate methods for such analyses.

The sensitivity of the various techniques applied is summarized in Table II.

Examination of Essential Oils from Other Mint Species

CGTC-analyses based on the experimental techniques described were also carried out on oil of spearmint (*M. cardiaca, M. spicata, M. viridis*), oil of pennyroyal (*M. pulegium* and *Hedeoma pulegioides*), oil of bergamot mint (*M. citrata*), oil of *M. sylvestris*, and oil of *M. rotundifolia*. The presence of menthofuran in each of these species was thus likewise established for the first time (see Table I).

BIOCHEMICAL SIGNIFICANCE OF EXPERIMENTAL RESULTS

The specificity of the metabolic pathways governing formation of carvone derivatives in spearmints and of menthone derivatives in peppermints was first pointed out by Kremers (19). Later, Reitsema substantiated and expanded these observations on the basis of a series of profound and extensive studies, grouping mint oils into the spearmints, peppermints, and lemon mints. Spearmints were characterized by the presence of 2-oxygenated *p*-menthanes, (carvone group), peppermints (including *M. arvensis* and *M. pulegium*) by the presence of 3-oxygenated *p*-menthanes (menthone group), and lemon mints by the presence of acyclic compounds. The coexistence of both 2- and 3-oxygenated *p*-menthanes in a given species was questioned and formation of one or the other of the three types considered exclusive (20). Subsequent workers acknowledged and endorsed this classification (21, 22). Recently, however, the occurrence of trace amounts of 3-oxygenated terpenoids, menthone and isomenthone, in spearmints was observed in this laboratory (14). The present study demonstrates the existence of a cyclic constituent, menthofuran, in *M. citrata*. Furthermore, the occurrence of pulegone, a possible precursor to menthofuran formation, was likewise established in this species by the CGTC technique. Small amounts of the cyclic ketone occurred also in *M. sylvestris* and *M. rotundifolia*. Its presence in *M. cardiaca, M. spicata, M. viridis*, and *M. arvensis* was previously reported (10, 14).

These observations appear to confirm that similar stereospecific biochemical processes are taking place in most, if not all, species of the genus *Mentha*, but at very different rates (23). A critical biogenetic scheme correlating the experimental data reported with others now assembled will make the subject of a forthcoming publication (24).

This study has shown how a delicate constituent not readily identified by direct application of classical chemical as well as modern instrumental methods of analysis may be detected and estimated in the various species of a given genus by means of the CGTC technique. In this regard it serves as a further example of the value of this method for the more thorough exploration of specific compositional regions comprising a gas chromatogram, for the isolation and determination of a minor constituent in a complex natural material, and hence for the establishment of new, sensitive criteria of product authenticity.

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