A Modified Method for the Rapid Analysis of Long-Chain Alkanes and Neophytadiene from Tobacco*

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INTRODUCTION

Among the major classes of compounds on the surface of the tobacco leaf are the aliphatic hydrocarbons, which consist primarily of C_{25}–C_{36} chain length compounds. Mold et al. (1) established that they consisted of a homologous series of normal (n), iso (i, 2-methyl, and anteiso (a, 3-methyl) compounds. These findings were later confirmed by gas chromatography — mass spectrometry (GC-MS) studies (2). Studies by Jenkins et al. (3, 4), Chortyk et al. (2), and Severson et al. (5) showed that the hydrocarbons are essentially transferred unchanged to smoke. Ivanov and Ognyanov (6) reported that the paraffinic hydrocarbons have a negative effect on smoke flavor and according to Davis (7) they are probably not related to leaf quality.
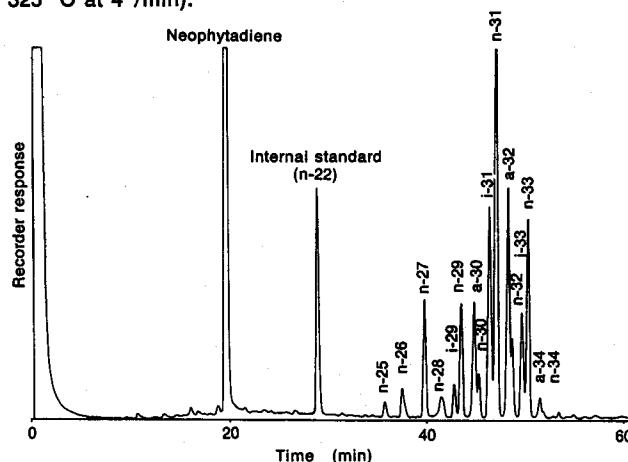
However, recent observations in our laboratory indicate that the hydrocarbons may be important leaf constituents. During lipid analyses of insect resistant and non-resistant tobaccos, we found that all analyzed tobaccos had essentially identical sterol and fatty acid distributions. However, each tobacco appeared to have its own characteristic hydrocarbon distribution. For example, several differences in distribution and in the ratios of normal to branched-chain alkanes were observed. For the flue-cured NC 2326 tobacco, we also observed that different crop years, leaf position, and fertilization practices had little, if any, effects on hydrocarbon distributions. Thus, hydrocarbon distribution may be indicative of a tobacco's genetic background and hydrocarbons could be used as chemotaxonomic markers in tobacco breeding work. Consequently, it was important that varietal differences in hydrocarbon distribution should be further investigated. However, such a study would require the analyses of numerous tobacco samples. Examination of existing methods of analyses indicated that they were too time-consuming to allow the examination of numerous samples and that new methodology needed to be developed.

Traditionally, hydrocarbons have been extracted from ground tobacco by hexane in a Soxhlet apparatus. After separation from interfering hexane-solubles by silicic acid (SA) column chromatography, they have been quantitated by conventional gas chromatography (GC) methods (2, 7, 8, 9). The lengthy Soxhlet extraction time (3—24 h) and the number of manipulations required for assembly and disassembly of the apparatus were obviously undesirable for daily analyses of many samples.

In our previous hydrocarbon analyses, the GC step involved the use of a packed Dexsil 300 GC column and required a long temperature program to separate the hydrocarbons (Figure 1). Also, this system partially resolved the normal from the methyl-branched isomers, but failed to yield any resolution of the iso and anteiso isomers. Consequently, this GC method also required improvements.

In this report, we describe two major modifications of the traditional hydrocarbon—neophytadiene analysis method: an ultrasonic vibration — extraction procedure using methylene chloride and analysis of the hydrocarbon—neophytadiene isolate by glass capillary GC. Both improvements greatly decreased analysis time, and the use of capillary GC permitted the separation and the quantitation of all of the hydrocarbon isomers.

Figure 1. Chromatogram of tobacco hydrocarbon isolate on a 4.6 cm × 6 mm stainless steel column packed with Dexsil 300 GC on 80/100 mesh Chromosorb W/AW (100–325 °C at 4°/min).

EXPERIMENTAL

Materials

All solvents used were Burdick and Jackson* "distilled-in-glass" grade and were redistilled in glass before use. The hydrocarbon standards were obtained from Analabs, Inc. and were checked for purity before use.

Tobacco Preparation and Dry Weight Determination

The NC 2326 tobacco used in this study was grown and cured, under conditions normally used for flue-cured tobacco, at the Tobacco Research Laboratory, Oxford, North Carolina, in 1978. The tobacco was ground to 32 mesh in a Wiley mill. For moisture determination, about 500 mg of ground tobacco was heated for 3 h at 95 ± 0.05 °C in a vented oven.

Methylene Chloride — Ultrasonic Vibration Extraction

About 0.5 g of tobacco was weighed into a 500 ml screw-top Erlenmeyer flask. To the flask were added 200 ml of CH_2Cl_2 and 1 ml of the internal standard solution [3-methyltricosane (a-C_{24}), 0.08 mg/ml in hexane]. The flask was placed in an ultrasonic vibration bath at room temperature for 15 min. The mixture was filtered through folded filter paper (Reeve Angel 802) into a 500 ml round-bottom flask. The flask, filter, and funnel were rinsed with 50 ml of hexane, and the resulting solution was reduced on a rotary evaporator at 30 °C and under house vacuum ($\approx$ 20 mm Hg) to a volume of about 2 ml. The residue was quantitatively transferred to a 10 ml volumetric flask with hexane (stock solution).

Hexane — Soxhlet Extraction

About 2 g of tobacco was placed into a paper extraction thimble (25 × 80 mm) and covered with a glass wool plug. Four milliliters of the internal standard solution (a-C_{24}, 0.08 mg/ml) and 200 ml of hexane were placed in a boiling flask and the tobacco was extracted for 8 h. The solvent was removed as above and the residue was quantitatively transferred to a 10 ml volumetric flask (stock solution).

Silicic Acid Chromatography

A Fisher and Porter 150 mm × 9 mm inside diameter chromatographic column equipped with a sintered glass disc, tapered stockcock, and a 250 ml globe-shaped reservoir was slurry-packed with 2.5 g of "Unisil" silicic acid (Clarkson Chemical Co., Williamsport, Pennsylvania, 100/120 mesh, activated at 150 °C for 2 h) in hexane. An aliquot of the stock solution (5 ml from the ultrasonic vibration extraction or 2 ml from the Soxhlet extraction) was placed on top of the column and the column was eluted with 50 ml of hexane into a 250 ml round-bottom flask. The column was operated under 3—5 p.s.i.** nitrogen pressure. The hexane fraction was concentrated on a rotary evaporator and the residue was transferred with hexane to a tapered test tube. The hexane was removed under nitrogen and the residue was redissolved in 100 μl of isooctane to yield the hydrocarbon-neophytadiene isolate.

Glass Capillary Gas Chromatography

Half (50 μl) of the above hydrocarbon isolate was placed in a microautoinjector vial, capped, and a 1 μl portion was analyzed in a Hewlett-Packard 5830 gas chromatograph, equipped with a Model 7671 A automatic sampler. Glass capillary GC separation was conducted on a 30 m × 0.25 mm inside diameter dynamically coated SE-54 glass capillary column (10) (linear flow velocity, 28 cm/s helium; split flow, 100 ml/min; oven temperature program, 160—280 °C at 4 °/min, injection port temperature, 300 °C; flame ionization detector temperature, 325 °C; helium make-up, 30 ml/min; hydrogen, 30 ml/min; air, 250 ml/min). The gas chromatograph was modified for use with glass capillary columns as previously described (11).

Component Identification and Quantitation

Neophytadiene and hydrocarbons were identified by conventional GC-MS methods, using a Dexsil 300 column, as previously reported (2). Identifications for the glass capillary GC separations were based on the retention times of the commercially available straight-chain hydrocarbons and the elution orders of the n-, i-, and a-isomers of C_{24}. Components were quantitated using the internal standard data reduction method supplied with the Hewlett-Packard 5830 GC system. Based on the fact that the n-, i-, and a-isomers of C_{24} yielded identical gas chromatographic responses, the branched isomers were assumed to yield GC responses identical to those of the commercially available straight-chain components. Neophytadiene was assumed to yield a GC response identical to phytane. Yields of neophytadiene and hydrocarbons were based on the amount of internal standard added to the sample before extraction.
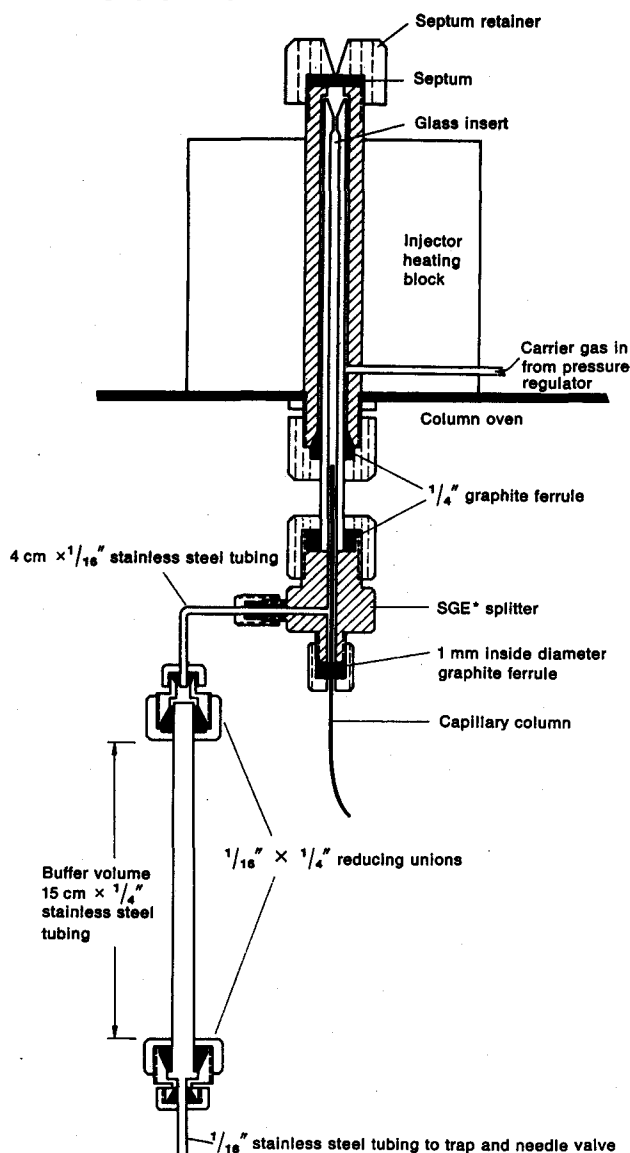
RESULTS AND DISCUSSION

In order to obtain more information on the level of each hydrocarbon isomer, we investigated glass capillary GC separation techniques. However, the commercial capillary injection system purchased with the gas chromatograph failed to yield reproducible and linear response data with standards. For hydrocarbons above C_{28}, the response dropped off dramatically and was very erratic and the system was not satisfactory for obtaining quantitative data. Therefore, we modified the standard on-column injection port as shown in Figure 2. A glass insert prepared from standard 6.4 mm × 2 mm inside diameter

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* Reference to a company or product name does not imply approval or recommendation by the United States Department of Agriculture.

** pound per square inch

Figure 2. Modified injector system for glass capillary gas chromatography analysis.

Labels in figure: Septum retainer; Septum; Glass insert; Injector heating block; Carrier gas in from pressure regulator; Column oven; $^1/_4''$ graphite ferrule; 4 cm × $^1/_{16}''$ stainless steel tubing; SGE* splitter; 1 mm inside diameter graphite ferrule; Capillary column; Buffer volume 15 cm × $^1/_4''$ stainless steel tubing; $^1/_{16}'' × ^1/_4''$ reducing unions; $^1/_{16}''$ stainless steel tubing to trap and needle valve

* Scientific Glass Engineering Pty., Ltd., Melbourne, Australia.

Pyrex glass tubing was secured to the end of the port with graphite ferrules. To the end of the tube we attached a commercially available splitter (Scientific Glass Engineering Pty., Ltd., Melbourne, Australia). The end of the capillary column was placed up into the insert, thus providing for an all glass system. A buffer volume was required to obtain linear response. Analyses were performed in the split mode of operation. A split flow of 100 ml/min was found to be optimum for linear split. The complete modification method is detailed elsewhere (11). Using this modification, we found that a 30 m SE-54 capillary column not only resolved the n-, i-, and a-isomers (Figure 3), but also reduced GC analysis time by about 50% compared to conventional, packed column GC.

Table 1 lists response reproducibility data obtained on the modified capillary system for a standard hydrocarbon mixture. As desired, we obtained essentially linear response from C_{20} to C_{34}, with relative standard deviations of less than 2%. Data on triplicate GC analyses of isolated tobacco hydrocarbons, using autoinjection, are given in Table 2. The data show that an acceptable capillary system had been constructed.

Since it is well documented that hydrocarbons are on the surface of the leaf (7, 12, 13), it appeared that an ultrasonic vibration solvent extraction of ground tobacco would be satisfactory to remove the hydrocarbons. Based on the addition of 3-methyltricosane to the tobacco samples, we found that 15 min ultrasonic vibration extraction of 0.5 g of ground tobacco with 200 ml of methylene chloride resulted in quantitative recovery of the tobacco hydrocarbons. Other solvent to tobacco ratios and extraction times were not investigated. Consequently, the scheme in Figure 4 was used to isolate the hydrocarbons and neophytadiene and obtain quantitative data. Capillary GC was used to compare extraction efficiencies of the standard Soxhlet and the ultrasonic vibration extraction methods. The data on reproducibilities for the methylene chloride ultrasonic vibration extraction method and for the traditional Soxhlet extraction procedure are given in Table 3. In order to reduce the variability of the data due to GC analysis (see Table 2), each set of data in Table 3 is the average of two GC determinations per replicate sample. Both methods resulted in the isolation of hydrocarbon fractions with essentially identical levels of individual and total hydrocarbons. Because of their low levels in leaf, C_{26} and C_{34} hydrocarbons yielded data that showed high relative standard deviations.

Since the ultrasonic vibration extraction step can be performed in less than half an hour compared to 8 h Soxhlet extractions, a large number of samples can be simultaneously extracted in one working day. Analysis time for

Table 2. Reproducibility of glass capillary gas chromatography analyses.

Component	Leaf percentage × 100*			
	run 1	run 2	run 3	Average ± percent rel. S.D.**
n-C_{25}	0.14	0.14	0.14	0.14 ± 0.0
n-C_{27}	0.99	1.01	0.99	1.00 ± 1.2
i-C_{30}	0.08	0.08	0.08	0.08 ± 0.0
a-C_{30}	1.00	1.08	1.06	1.05 ± 4.0
n-C_{30}	0.41	0.44	0.44	0.43 ± 4.0
i-C_{31}	1.54	1.72	1.60	1.62 ± 5.7
a-C_{31}	0.28	0.31	0.30	0.30 ± 5.1
n-C_{31}	3.66	4.09	4.03	3.93 ± 5.9
i-C_{32}	0.13	0.15	0.14	0.14 ± 7.1
a-C_{32}	1.47	1.68	1.62	1.59 ± 6.8
n-C_{32}	0.63	0.72	0.68	0.68 ± 6.7
Total C_{25}–C_{34}	14.5	15.6	15.3	15.13 ± 3.8
Neophytadiene	13.5	13.6	13.6	13.57 ± 0.4

* Calculated assuming unitary detector response.
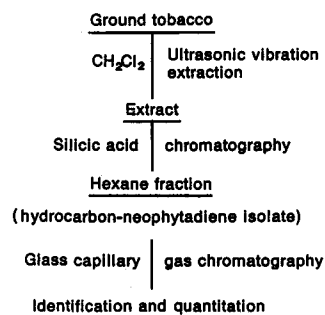** Relative standard deviation = S.D./mean × 100.

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Table 1. GC peak identifications and response data on hydrocarbons.

Peak No.	Compound	Response factor (K)* ± percent rel. S.D.**
	Phytane	0.99 ± 1.1
1	Neophytadiene	
2	$a\text{-}C_{24}$	1.00
	$n\text{-}C_{24}$	0.99 ± 0.4
3	$n\text{-}C_{25}$	0.99 ± 0.4
4	$n\text{-}C_{26}$	0.99 ± 0.6
5	$a\text{-}C_{27}$	
6	$n\text{-}C_{27}$	0.99 ± 0.4
7	$i\text{-}C_{28}$	
8	$a\text{-}C_{28}$	
9	$n\text{-}C_{28}$	0.99 ± 0.8
10	$i\text{-}C_{29}$	
11	$a\text{-}C_{29}$	
12	$n\text{-}C_{29}$	
13	$i\text{-}C_{30}$	
14	$a\text{-}C_{30}$	
15	$n\text{-}C_{30}$	0.99 ± 1.7
16	$i\text{-}C_{31}$	
17	$a\text{-}C_{31}$	
18	$n\text{-}C_{31}$	1.05 ± 1.2
19	$i\text{-}C_{32}$	
20	$a\text{-}C_{32}$	
21	$n\text{-}C_{32}$	1.04 ± 1.7
22	$i\text{-}C_{33}$	
23	$a\text{-}C_{33}$	
24	$n\text{-}C_{33}$	
25	$i\text{-}C_{34}$	
26	$a\text{-}C_{34}$	
27	$n\text{-}C_{34}$	1.05 ± 1.3
28	$i\text{-}C_{35}$	
29	$n\text{-}C_{35}$	
30	$a\text{-}C_{36}$	
31	$n\text{-}C_{36}$	

* K = (weight compound / area compound) / (weight standard / area standard).
** Relative standard deviation = S.D./mean × 100.

Figure 4. Hydrocarbon analysis method.

Ground tobacco
CH_2Cl_2 | Ultrasonic vibration extraction
Extract
Silicic acid | chromatography
Hexane fraction
(hydrocarbon-neophytadiene isolate)
Glass capillary | gas chromatography
Identification and quantitation

Figure 3. Capillary chromatogram of tobacco hydrocarbon isolate on a 0.25 mm × 30 mm SE-54 wall-coated open tubular column (160-280 °C at 4°/min). (See Table 1 for peak identifications.)
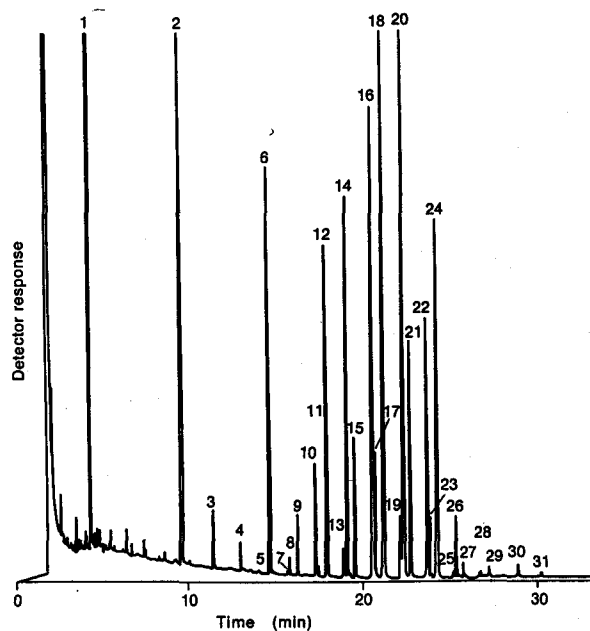
Table 3. Comparison of CH$_2$Cl$_2$ ultrasonic vibration and hexane — Soxhlet extraction methods.

Compound	CH$_2$Cl$_2$ - ultrasonic vibration				Hexane-Soxhlet			
	replicate 1	replicate 2	replicate 3	average ± rel. S.D.	replicate 1	replicate 2	replicate 3	average ± rel. S.D.
*Percentage distribution**								
C$_{25}$**	0.78	0.78	0.83	0.80 ± 3.6	0.75	0.70	0.71	0.72 ± 3.7
C$_{26}$	0.39	0.38	0.56	0.44 ± 22.8	0.45	0.36	0.33	0.36 ± 16.4
C$_{27}$	5.66	5.66	5.68	5.67 ± 0.2	5.55	5.40	5.37	5.44 ± 1.8
C$_{28}$	1.31	1.28	1.29	1.29 ± 1.2	1.13	1.17	1.09	1.13 ± 3.5
C$_{29}$	9.24	9.19	9.24	9.22 ± 0.3	9.01	9.06	8.98	9.02 ± 0.4
C$_{30}$	9.77	9.75	9.86	9.79 ± 0.6	9.42	9.33	9.36	9.37 ± 0.5
C$_{31}$	39.19	39.47	39.87	39.51 ± 0.9	40.50	39.55	40.92	40.32 ± 1.7
C$_{32}$	17.01	16.91	16.86	16.93 ± 0.5	16.87	16.48	16.95	16.77 ± 1.5
C$_{33}$	15.41	15.13	14.84	15.13 ± 1.9	16.65	15.25	15.31	15.40 ± 1.4
C$_{34}$	1.25	1.04	0.97	1.09 ± 13.4	0.72	0.88	0.73	0.78 ± 11.5
Leaf percentage+								
Total hydrocarbons	0.188	0.185	0.183	0.185 ± 0.6	0.188	0.189	0.190	0.189 ± 0.5
Neophytadiene++	0.131	0.139	0.133	0.134 ± 3.1	0.136	0.144	0.144	0.141 ± 3.3

* Based on total hydrocarbon area count; areas corrected for difference in detector response; methyl–branched isomer assumed to respond identically with straight-chain isomer; average of two GC determinations on each replicate sample.

** Total n-, i-, and a-isomers.

+ Based on dry weight.

++ Assumed to respond identically with phytane.

capillary GC is about half of that for packed column GC. The use of an automatic sample injection assembly also allows the handling of a large number of samples. Consequently, both modifications have greatly facilitated and shortened the hydrocarbon analysis. This rapid method for leaf hydrocarbons will now be used to screen numerous tobacco variety samples to test our hypothesis that hydrocarbon distribution may be characteristic of a tobacco's genetic background.

SUMMARY

A rapid method for the analysis of aliphatic hydrocarbons and neophytadiene in cured tobacco was developed. Briefly, ground tobacco was extracted for 15 min with methylene chloride in a flask placed in an ultrasonic vibration bath. 3-Methyltricosane was added as the internal standard. The solution was filtered and the solvent removed. The sample was redissolved in hexane and chromatographed on a small silicic acid column. The hydrocarbon-neophytadiene fraction that eluted with hexane was reduced in volume and analyzed on a SE-54 wall-coated glass capillary column, which resolved and permitted quantitation of the *normal*, *iso*-methyl-branched, and *anteiso*-methyl-branched hydrocarbons. The analytical data obtained by this method were equal to those obtained by the conventional Soxhlet extraction procedure. Based on experience in our laboratory, analysis time was reduced about fivefold and isomeric hydrocarbons were resolved by capillary gas chromatography.

ZUSAMMENFASSUNG

Zur Bestimmung von aliphatischen Kohlenwasserstoffen und Neophytadien in getrocknetem Tabak wurde ein Schnellverfahren entwickelt, das aus folgenden Analyseschritten besteht: Gemahlener Tabak wurde 15 min lang mit Dichlormethan in einem Ultraschallvibrationsbad unter Zugabe von 3-Methyltrikosan als innerem Standard extrahiert. Nach Filtrierung der Lösung und Entfernung des Lösungsmittels wurde das Probegut erneut in Hexan gelöst und chromatographisch auf einer kleinen Kieselsäure-Säule aufgetrennt. Die die Kohlenwasserstoffe und Neophytadien enthaltende Fraktion, die mit Hexan eluiert wurde, wurde eingedampft und gaschromatographisch unter Einsatz einer mit SE-54 beschichteten Glaskapillarsäule untersucht, wodurch die Auflösung und die quantitative Bestimmung von *unverzweigten*, *iso*-methylverzweigten und *anteiso*-methylverzweigten Kohlenwasserstoffen erreicht wurde. Mit diesem Verfahren wurden die gleichen Analysewerte erhalten wie mit der herkömmlichen Soxhlet-Extraktionsmethode. Die praktische Anwendung der Methode zeigte, daß bei diesem neuen Verfahren die Analysendauer auf ein Fünftel verkürzt werden kann, wozu noch der Vorteil einer differenzierteren Aussage durch die Auftrennung der isomeren Kohlenwasserstoffe mit Kapillargaschromatographie kommt.

RÉSUMÉ

Une méthode rapide pour l'analyse des hydrocarbures aliphatiques et du néophytadiène dans le tabac séché a

été mise au point. La méthode opère comme suit: Le tabac moulu est extrait avec du chlorure de méthylène pendant 15 minutes dans un flacon placé dans un bain à ultra-sons avec adjonction de 3-méthyltricosane comme référence interne. Après filtrage de la solution et évaporation du solvant, le résidu est redissous dans de l'hexane et chromatographié sur une petite colonne d'acide silicique. La fraction contenant les hydrocarbures et le néophytadiène, éluée avec l'hexane, est concentrée et chromatographiée sur une colonne capillaire de verre imprégnée de SE-54, résultant dans la séparation et la détermination quantitative des hydrocarbures *normaux*, *iso*-méthylés et *anteiso*-méthylés. Les résultats obtenus par cette méthode sont identiques à ceux obtenus par le procédé d'extraction conventionnel au Soxhlet. L'expérience des auteurs démontre, en outre, que la nouvelle méthode ne requiert qu'un cinquième du temps, sans compter l'avantage de la séparation des hydrocarbures isomères par la chromatographie sur colonne capillaire.

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