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Determination of Adulterated Natural Ethyl Butyrate by Carbon Isotopes

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Ethyl butyrate, a flavor chemical found in many foods, was isolated from microbial sources and orange juice. The two authentic "natural" samples and one synthetic sample were analyzed for ^{14}C content. The natural samples yielded values consistent with their natural origin (ca. 125% of modern), while the synthetic sample was devoid of ^{14}C, as expected for a petrochemical material. From the carbon stable isotope ratio, it was possible to differentiate between the two types of natural ethyl butyrate, but this technique proved less useful in distinguishing between "natural" and "artificial" material.

The use of ^{14}C analysis in the food industry for determining sources of ethanol was first suggested by Faltings (1952). It has since been applied to fermented spirits (Martin et al., 1981; McWeeney and Bates, 1980), vinegar (Krueger and Krueger, 1985), caffeine (Allen, 1961), cinnamon (Hoffman and Salb, 1980), citric acid (Volpe et al., 1982), and other flavor chemicals (Devron et al., 1980; Bricout and Koziet, 1978).

Ethyl butyrate is an important flavor chemical found in many foods. It is most abundant in fruit juices such as orange, apple, and strawberry. Foods are often formulated to taste like the above flavors by the addition of "natural" and/or "artificial" flavors, with a corresponding claim on the product label. Artificial flavors are usually made from inexpensive and abundant petrochemical sources, while natural flavors are generally expensive and in limited supply. With the current consumer trend toward natural foods, there is a possibility for fraud by adulterating natural flavorings with inexpensive artificial flavoring materials.

Research in our lab has centered on obtaining ethyl butyrate from sources that comply with the Code of Federal Regulations (21 CFR 101.22.a.3) for natural flavors. In light of the above legal and economic aspects, it was important to have an analytical method that would distinguish between natural and artificial ethyl butyrate. We report in this paper the use of ^{14}C analysis as a means to identify ethyl butyrate obtained from petrochemical sources.

MATERIALS AND METHODS

Samples of authentic natural ethyl butyrate were prepared by standard methods of extraction and distillation (Kesterson and Braddock, 1976) to obtain Food Chemical Codex quality product. Artificial ethyl butyrate was purchased from Fritzsche Dodge & Olcott Inc. The samples were coded and submitted blind to the analysts.

^{14}C Analysis. Approximately 5 g of sample was placed in a 2-L combustion bomb and the bomb charged with 100 psi of O_2. The sample was ignited electrically. When the pressure surge subsided and the bomb cooled, the resultant gases were passed through traps of dry ice/trichloroethylene and liquid O_2. *Caution*! Proper precautions must be observed in the handling of liquid O_2, which is used instead of liquid N_2 to avoid O_2 condensation in the gas line. The liquid O_2 trap was isolated and any residual noncondensible gas evacuated. An aliquot of the CO_2 was taken for ^{13}C analysis and the remaining CO_2 converted to methane with tritium-free H_2 over 0.5% ruthenium on alumina at 475 °C. The methane was purified by passing through a trap of dry ice/trichloroethylene, trapping on silica gel at liquid N_2 temperature, and evacuating excess H_2. Methane was released by warming the silica gel trap and freezing the evolved methane in a liquid N_2 trap and expanded into a storage flask to await ^{14}C counting. The ^{14}C activity of methane was determined by counting for 1000 min in a low-level proportional gas counter with anticoincidence circuitry, which was calibrated using the NBS oxalic acid standard. Counter background was measured using a 300-million-year-old marble with no ^{14}C activity. ^{14}C activities were corrected for isotope fractionation by normalizing to $^{13}C = -25.0‰$ using

$$^{14}C_{norm} = {}^{14}C_{meas}\left[1 - \frac{2[25 + \delta(^{13}C)]}{1000}\right]$$

^{13}C Analysis. CO_2 was analyzed to determine its $^{13}C/^{12}C$ ratio by using a dual collecting mass spectrometer according to AOAC (13th ed., 31.153, 1980).

RESULTS AND DISCUSSION

^{14}C Analysis. Carbon-14, present in the atmosphere mainly as $^{14}CO_2$, is produced in nature by cosmic radiation (Friedlander and Kennedy, 1962). The relative abundance

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Table I. Known and Projected ^{14}C Activities[a]

year	atm CO_2 ^{14}C act.,[b] % modern	year	atm CO_2 ^{14}C act.,[c] % modern
1975	139	1981	127
1976	136	1982	126
1977	134	1983	124
1978	133	1984	123
1979	131	1985	121
1980	128	1986	120

[a] Krueger and Krueger (1985). [b] The standard deviation of recent atmospheric ^{14}C measurements is approximately 2% of modern for any given year. [c] Projections based on a best fit model of first-order mixing of atmospheric CO_2 with a large excess of oceanic carbonate of 100% modern composition. $^{14}C_t = 100 \pm 49.1e^{-0.0646(t-1972)}$.

Table II. Carbon Isotope Measurements on Ethyl Butyrate

sample	origin[a]	^{13}C,[b] %	^{14}C act.,[c] % of modern[d]
1	microbiological (corn syrup glucose)	−14.7	121.4
2	orange juice	−29.6	129.1
3	artificial	−24.7	1.9

[a] Coded samples submitted blind to analyst. [b] Standard deviation ±0.1%. [c] Standard deviation ±2%. [d] 100% of modern equals 95% of the activity of NBS oxalic acid standard or 13.56 dpm/g of carbon.

of ^{14}C in nature is approximately 1.3×10^{-12}, with respect to the stable isotope ^{12}C (Allen, 1961). Plants incorporate ^{14}C from atmospheric carbon dioxide through photosynthetic carbon fixation, with little preferential absorption. When an organism dies, it ceases to fix carbon. Carbon-14 is an unstable isotope, decaying with a half-life of 5670 years (Weast, 1980).

The ^{14}C content of terrestrial plants was approximately 100% of the "modern standard" activity when the standard was established in 1950. Since the early 1960s, the level of atmospheric ^{14}C has been elevated due to nuclear weapons testing, reaching a peak in 1964 and 1965. With the advent of the Limited Test Ban Treaty, the levels are slowly declining back toward 100%. Because the atmospheric ^{14}C content is well documented (Nydal and Lovseth, 1983), the amount of ^{14}C in a plant grown in a given year can be accurately predicted. Table I lists the recent ^{14}C values for atmospheric CO_2, as well as projections for the next few years. Similarly, on the basis of their ^{14}C content, chemicals extracted or otherwise isolated from plants can be "dated" to the approximate year in which the plant (or plant part) was grown.

Chemicals of petrochemical origin have had sufficiently vast periods of time to allow all the ^{14}C to decay until depleted. Since products from recent plant sources will have ^{14}C values of about 125% of the standard for modern activity, it is possible to distinguish this material from petroleum based chemicals. Natural chemicals, having been isolated from recently fixed carbon sources, can therefore be distinguished from petrochemicals or from their mixtures with natural chemicals.

The results of our ^{14}C analysis (Table II) show that the natural ethyl butyrate isolated in our laboratory from microbial sources (sample 1) and orange juice (sample 2) have modern values; they are from recently fixed carbon sources. The sample of artificial ethyl butyrate (sample 3) had negligible ^{14}C.

While it is possible to distinguish petroleum and recently fixed carbon samples, ^{14}C analysis presents one disadvantage. It cannot distinguish between a natural (recent carbon source) chemical and a substance chemically synthesized from natural (recent carbon source) chemicals. An example is citral, normally found in lemon oil, which can be synthesized from turpentine. The synthesized citral, although artificial, would be indistinguishable from the natural citral by ^{14}C content (Bricout and Koziet, 1978).

^{13}C Analysis. It is expected that ethyl butyrate derived from coal or petroleum feedstocks would give carbon stable isotope ratio analysis (SIRA) values in the range of about −24 to −30‰, as this is the range for the starting feedstocks (Faure, 1977). The SIRA values obtained for natural ethyl butyrate are expected to vary according to the natural source material; ethyl butyrate derived from Hatch–Slack (C_4) photosynthetic plant material should give values near −10‰, while that derived from Calvin cycle (C_3) plant material should give values near −25‰.

We observed (Table II) that the SIRA values for natural and synthetic ethyl butyrate were in line with expectations. Ethyl butyrate isolated from orange (C_3 plant) gave an SIRA value of −29.6‰, while that derived from corn syrup (C_4 plant) gave an SIRA value of −14.7‰. It has been observed that fatty acids and other reduced carbon materials in plants tend to be more negative than sugars from the same plant (Whelan et al., 1970). The present results are consistent with such observations. The synthetic ethyl butyrate samples gave results within the expected range. It is clear that SIRA will be of limited usefulness in the quality control of ethyl butyrate. The SIRA values of C_3 plant and petrochemical ethyl butyrate are similar enough that the two cannot be reliably distinguished. SIRA can readily distinguish between fermentation ethyl butyrate (from corn syrup feedstock) and other butyrates, natural or synthetic.

CONCLUSION

The determination of ^{14}C in natural ethyl butyrate constitutes a very sensitive means of detecting adulteration with synthetic material from petrochemical sources. The large difference between natural and synthetic values should allow for the detection of small quantities of added synthetic material.

Carbon SIRA values, which are obtained as a byproduct of the ^{14}C determination, are of limited use in ethyl butyrate quality control. Ethyl butyrate from petrochemical sources cannot be readily distinguished from that of C_3 plant sources. Ethyl butyrate from C_4 sources can be readily distinguished from other types of natural and synthetic ethyl butyrate.

Registry No. ^{14}C, 14762-75-5; ethyl butyrate, 105-54-4.

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Byproduct Identification in the Carbodiimide-Assisted Synthesis of Fatty Acid Anilides Related to Spanish Toxic Oil Syndrome

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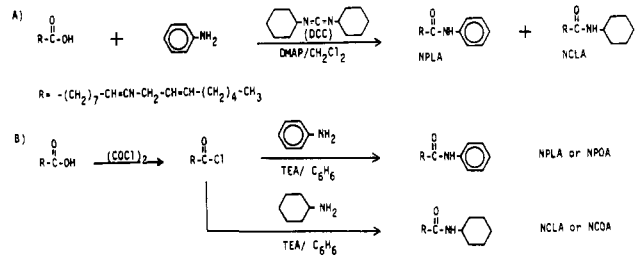
Results are presented on the comprehensive analytical identification of the *N*-cyclohexylamides obtained as unexpected reaction byproducts in the synthesis of *N*-phenyloleamide (NPOA) and *N*-phenyllinoleamide (NPLA) by reaction of anilide with either oleic or linoleic acid. The data presented prove that these *N*-cyclohexylamide impurities (5–25%) are due to the *N,N'*-dicyclohexylcarbodiimide used as dehydrating agent. The synthesis of both phenylamides was required for toxicological testing in relation to the Spanish Toxic Oil Syndrome. Purification of the reaction crudes by silica gel column chromatography followed by subsequent TLC and HPLC assay of the presumably pure amides did not eliminate the cyclohexylamides, which had not been detected by either technique. Unequivocal identifications were achieved by a combined analytical approach involving packed and capillary GC and GC–MS, chemical ionization MS, IR and NMR spectroscopy, and elemental analysis. On the basis of the above results, an alternative procedure is suggested, leading to the desired amides in high purity and good yields.

INTRODUCTION

The anilides of oleic and linoleic acid have recently attracted much interest for their presumed implication in the mechanism(s) of toxicity underlying a new epidemic disease that has become known as the Spanish Toxic Oil Syndrome (TOS) (Tabuenca, 1981; Pestaña and Muñoz, 1982; Toxic Epidemic Syndrome Study Group, 1982; Gelpi, 1985). These compounds—which are formed by reaction of aniline with the free fatty acids in edible oils—were found in significant amounts in rapeseed oil batches. Rapeseed oil for industrial uses had been originally denatured with 2% aniline, but later it was subjected to a fraudulent and unsuccesful refining process with the aim of making it suitable for human consumption (Granjean and Tarkowski, 1984). For this purpose it was also mixed with other varieties of food oils and animal fats. The abatement of the epidemic disease caused by these illegal oils coincided with their withdrawal from the market by the Spanish authorities as soon as a reasonable epidemiological link was established (Granjean and Tarkowski, 1984).

Thus, from the beginning, the anilides of oleic and linoleic acids were the most likely candidates for detailed analytical and toxicological studies in the search of the ethiopathogenesis of TOS. The anilides of oleic and linoleic acids have been referred to either collectively as "oleoanilides" or specifically as oleyl- and linoleylanilides in the literature related to the TOS. In compliance with accepted nomenclature, these compounds will be designated herein as indicated in Scheme I.

Scheme I. Synthesis Procedures for the Preparation of Fatty Acid Anilides



R = $-(CH_2)_7-CH=CH-CH_2-CH=CH-(CH_2)_4-CH_3$ or $-(CH_2)_7-CH=CH-(CH_2)_7-CH_3$
NPLA: *N*-phenyllinoleamide; NPOA: *N*-phenyloleamide; NCLA: *N*-cyclohexyllinoleamide; NCOA: *N*-cyclohexyloleamide

Different samples of oils presumably related to clinical case histories have been screened for the presence and content of these anilides by both chromatographic and mass spectrometric techniques (Artigas et al., 1983; Granjean and Tarkoski, 1984). Also they were subjected to various in vitro and in vivo toxicological testing procedures (Granjean and Tarkowski, 1984). All of this work has created the need for the availability of the pure anilides as reference compounds, as well as for relatively large amounts of the same compounds that are needed in animal model testing.

Consequently, several laboratories, including the Instituto Nacional de Toxicología, Madrid, attempted the synthesis of pure ole- and linoleanilides. Some of these syntheses have been reported in the literature (Casals et al., 1983; Fernandez-Alvarez, 1983). However, the laboratories that prepared these anilides, either for their own

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