Authentication of Mustard Oils by Combined Stable Isotope Analysis (SNIF-NMR and IRMS)

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Allyl isothiocyanate is the major component of the mustard oil. This molecule can be synthesized at a much lower price than its cost when extracted from mustard seeds. Adulteration of natural mustard oil by adding synthetic allyl isothiocyanate is therefore very profitable. In order to detect such a fraud, a new analytical approach has been developed using both SNIF-NMR (site-specific natural isotope fractionation studied by nuclear magnetic resonance) and IRMS (isotopic ratio mass spectrometry). This approach enables an unambiguous distinction between natural and synthetic allyl isothiocyanate. It also makes it possible to identify the geographical origin of natural mustard oils. The principle of the interpretation is based on a multivariate analysis of the isotopic parameters of allyl isothiocyanate, $(D/H)_i$, $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$, which provide largely independent information. The discriminating performances of the different isotopes are discussed.

Keywords: *Allyl isothiocyanate; mustard oil; SNIF-NMR; ^{13}C-, ^{15}N-, ^{34}S-IRMS; authentication; adulteration; geographical origin*

I. INTRODUCTION

Stable isotope methods are very useful techniques for characterizing the authenticity of food products. The first applications relied on isotope ratio mass spectrometry (IRMS). Some examples include those of Bricout et al. (1981), Butzenlechner et al. (1989), Hoffman and Salb (1979), Martin et al. (1983), and Doner et al. (1992, 1987).

Since the early 1980s the SNIF-NMR method (trademark of EUROFINS Laboratories) (Martin and Martin, 1981) has been shown to be a very powerful analytical tool for verifying the botanical and synthetic origin of flavor molecules (Martin et al., 1993). The distinction between synthetic or natural origin has been successfully achieved for several products leading to the publication of a number of papers dealing with the authenticity of vanillin (Maubert et al., 1988), benzaldehyde (Remaud et al., 1992b; Hagedorn, 1992), maple syrup (Martin et al., 1996b), fruit juices (Martin et al., 1996a), etc. In some cases, the botanical species of the plant from which the natural product was extracted may also be identified (linalool (Hanneguelle et al., 1992), acetic acid (Remaud et al., 1992a), ethanol (Martin and Martin, 1995), anethole (Martin et al., 1982)).

According to our knowledge no analytical methods are available to authenticate natural allyl isothiocyanate. Because the price of allyl isothiocyanate ex-mustard seeds is higher than that of its synthetic counterpart there is substancial economic interest to add synthetic product to natural mustard oil (Arctander, 1960; Clark, 1992). There are two types of mustard: black mustard and white mustard. Only black mustard gives an essential oil. Two species of black mustard are commonly used to produce essential oils: *Brassica nigra* and *Brassica juncea*. The latter is nowadays the main source of mustard seeds, producing an essential oil on distillation. Two countries, Canada and India, are currently trading the majority of commercial mustard seeds, the former being the world's larger producer of condiment mustard. Canada produces yellow-seeded *B. juncea* which is commonly known as "Oriental mustard". There are different varieties of Oriental mustard, such as Lethbridge 22A, Cutlass, and AC Vulcan. Canada also produces brown-seeded *B. juncea* which is commonly known as "brown mustard" (Rakow, 1997).

As a more thorough complement to a previous short communication on this topic (Martin et al., 1994), the aim of this paper is to discuss the potential of the SNIF-NMR method as a routine analytical tool to discriminate between the different origins of allyl isothiocyanate as well as to introduce $\delta^{34}S$ analyses of this material. Not only the distinction between allyl isothiocyanate generated from mustard seeds and synthetic allyl isothiocyanate but also the quantification of mixtures of the above are investigated. A multiisotopic strategy has been elaborated. Thus in addition to the overall $\delta^{13}C$ parameter, and to the site-specific $(D/H)_i$ ratios, the $\delta^{15}N$ and $\delta^{34}S$ deviations of allyl isothiocyanates from several origins have been determined. The authentication aptitudes of these parameters are estimated, and the interest of combining their various degrees of independent information for solving problems of increasing complexity is discussed. In particular the possibility of identifying the geographical origin of natural allyl isothiocyanate is investigated. It is shown that as ^{18}O (Dunbar and Wilson 1982), the stable isotopes ^{15}N and ^{34}S may be useful complementary sources of information. Some data concerning the use of $\delta^{15}N$ for characterizing the geographical origin of organic molecules have already been published (Danho et al., 1992; Kornexl et al., 1996), but until now the $\delta^{34}S$ deviation measured by mass spectrometry was mainly applied to mineral molecules; when organic molecules were used, the sulfur was first converted to $BaSO_4$ and later to SO_2

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by different techniques (Ecological Studies, 1988; Giesemann et al., 1985; Krouse and Levinson, 1984; Krouse and Case, 1981; Hirner et al., 1984; Krouse et al., 1987). The present paper pioneers the use of the ^{34}S content of organic molecules as a tool for origin assignment.

II. EXPERIMENTAL PROCEDURES

(a) Nature and Origin of the Products. Authentic samples were collected for all main origins in order to be used as references. Natural allyl isothiocyanates from Canada and India are commercially available. Some of the samples used as references for natural origins have been extracted directly from the seeds by a bench procedure while the others were collected from industrial processing plants. Synthetic allyl isothiocyanate samples were commercially obtained from Kodak, Riedel de Häen, Lancaster, Merck, Fluka, Aldrich, etc.

(b) Purification: Production of Pure Allyl Isothiocyanate. Mustard oil consists almost entirely of allyl isothiocyanate, which exists as a glycoside located in certain cells as in the case of vanilla (glucovanillin; Clark, 1990), bitter almond (amygdalin; Clark, 1995), and safranal (picrocrocin; Remaud et al., 1993). After hydrolysis, the essential oil is obtained by water steam distillation.

In a first approach the extraction of allyl isothiocyanate has been performed using the following procedure: (i) Hydrolysis of the glucoside present in the seeds in water at 60 °C for ~2 h; (ii) steam distillation; (iii) extraction of allyl isothiocyanate from water with ether; and (iv) distillation of ether.

Allyl isothiocyanate is toxic, flammable and a strong lachrymator. Great care must therefore be taken when handling it.

All these steps have to be performed under a fume hood, because of the toxicity of allyl isothiocyanate. The purity obtained is above 98%. The industrial process does not include solvent extraction, but there is a final distillation under vacuum. The last step cannot be reproduced in a lab-scale experiment. There are small isotopic differences between the industrial product and the bench samples. However these differences are negligible as compared to between samples variability. Therefore these differences cannot lead to erroneous conclusions.

(c) Deuterium NMR Spectroscopy. As a result of the low natural abundance of ^2H with respect to ^1H, the probability of observing bideuterated species is very low. Therefore, at the natural abundance level the ^2H-NMR spectrum in the presence of proton decoupling is composed of a single line for each monodeuterated species. The four monodeuterated isotopomers of allyl isothiocyanate are separately observed with a specific ^2H probe using high-field NMR spectrometers (Figure 1). The chemical shifts, expressed in ppm, are referred to TMS (tetramethylsilane, set at 0 ppm).

The site-specific hydrogen isotope ratio determinations were performed on a AM500 Bruker NMR spectrometer at probe temperature of 308 K. The spectra were recorded at 76.77 MHz using a specific 10 mm o.d. probe equipped with a ^{19}F locking device. A broad band proton decoupling was applied continuously. About 1 mL of pure allyl isothiocyanate was used. This relatively large amount of compound required special caution with regard to the handling for NMR. Teflon sample tube liners have to be placed inside the NMR tube to prevent glass breaking and toxic leakages. This inner tubing is detrimental to the resolution and peak

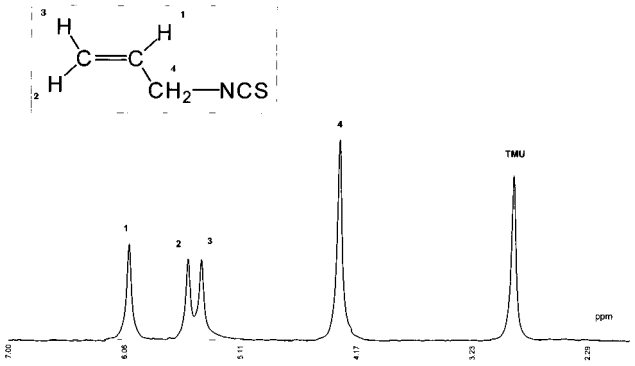

Figure 1. ^2H-NMR spectrum of synthetic allyl isothiocyanate. The four isotopomers have been assigned on the basis of the ^1H-NMR spectrum. Tetramethylurea (TMU) is used as a reference for calculating the site-specific deuterium contents $(D/H)_i$.

shape, although these disadvantages are partly compensated by the signal processing performed on the ^2H-NMR spectra. The phasing and base line corrections require great care since misadjustments can lead to erroneous and nonreproducible $(D/H)_i$ values. We have recently developed a new processing (EUROSPEC-LISS software) which applies a complex least squares curve-fitting analysis which automatically iterates over the base line and phase parameters and optimizes the quantitative determinations in terms of both precision and accuracy (Martin, 1994). An internal reproducibility better than 4% is now routinely achieved on flavoring molecules when using this procedure. The site-specific isotope distribution can be described by three types of parameters:

(i) The molar fractions of the monodeuterated isotopomers, f_i, are directly calculated from the signal area, S_i.

(ii) The relative parameters

$$R_{i/j} = F_j S_i / S_j \quad (1)$$

represent the number of deuterium atoms in site i with respect to site j, which is arbitrarily given its stoichiometric number of hydrogens, F_j. The parameters $R_{2/1}$, $R_{3/1}$, and $R_{3/2}$ are therefore the deuterium probability factors of site 2 and 3 in a situation characterized by the stoichiometric number $F_j = 1$ for the chosen reference site $j = 1$ or 2. For a random distribution of deuterium the three relative ratio $R_{i/j}$ would be equal to unity,

(iii) The site-specific isotope ratios $(D/H)_i$, expressed in ppm, are obtained from the SNIF-NMR spectra by using an internal referencing procedure (Guillou et al., 1993; Martin and Martin, 1995). The signal area of the investigated product are compared to that of an official reference, tetramethylurea (TMU), of known deuterium content added to the sample. This reference may be purchased from the Institute for Reference Materials and Measurements in Geel, Belgium.

(d) ^{13}C, ^{15}N, and ^{34}S IRMS Determinations. Isotopic contents are expressed as isotopic deviations δ defined as

$$\delta\ (‰) = (R_i/R_{\mathrm{ref}} - 1) \times 1000 \quad (2)$$

where R_i is the isotopic ratio of the whole molecule or of the molecular site i and ref that of the reference (Craig, 1953). The carbon and nitrogen results obtained

Table 1. Hydrogen Isotope Distribution Measured by SNIF-NMR on Allyl Isothiocyanate Extracted from Mustard Seeds (Natural) and Produced by Chemical Means (Synthetic)[a]

origin		$R_{2/1}$	$R_{3/2}$	$R_{3/1}$
natural	mean	0.86	0.93	0.80
	(n = 15) SD	0.03	0.04	0.02
synthetic	mean	1.06	0.99	1.02
	(n = 9) SD	0.03	0.02	0.02

[a] The relative parameters $R_{i/j}$ are defined in eq 2. SD: standard deviation of the mean.

by IRMS are expressed in δ (‰) with respect to the PDB (Pee Dee Belemnite) international standard for carbon and atmospheric nitrogen for nitrogen. The δ^{34}S (‰) isotopic deviation is referred to the international standard CDT (Canyon Diablo Troilite), after appropriate corrections (Eriksen et al., 1994).

The mass spectrometric determinations of the C, N, and S isotope ratios were carried out by on-line analysis using a Carlo-Erba NA 1500 II elemental analyzer fitted to a Fisons Instruments Optima mass spectrometer. The reagents used for δ^{34}S determinations were from Carlo-Erba.

Samples placed in tin containers were submitted to a flash combustion in a stream of helium enriched with pure oxygen. A conventional procedure was used for carbon and nitrogen determinations (Barrie and Lemley, 1989). The ^{34}S content was measured in a similar manner but with different experimental conditions. Only one reactor was used according to procedures previoulsy described (Giesemann et al., 1994; Pella and Colombo, 1978). The quartz reactor was filled in its upper part either with tungstic oxide (Fisons Instruments) or with tungstic oxide on alumina (Microanalysis). The lower part of the reactor was filled with "copper-reduced pure wires" (Fisons Instruments). Quantitative conversion into SO_2 was achieved by adjusting reactor temperature, oxygen concentration, and helium flow. The complete procedure and the isotopic reliability will be described separately in a publication dedicated to the analytical methodology (Naulet et al., 1997). The total internal reproducibility was better than 0.2‰ for carbon and nitrogen and 0.3‰ for sulfur.

III. RESULTS AND DISCUSSION

(a) Site-Specific Hydrogen Isotope Ratios of Mustard Oil. From the quantitative point of view, the ^{2}H-NMR spectrum of synthetic allyl isothiocyanate is very different from that of the naturally occurring species, in terms not only of the internal repartition but also of the total amount of deuterium (Tables 1 and 2).

In the synthetic species deuterium is almost equally distributed among the four molecular sites. This behavior is in agreement with a fossil source of hydrogens and with the set of chemical reactions involved to produce this compound. On the other hand, very large deviations with respect to a statistical distribution of deuterium are observed in the natural product and the peak pattern of the ethylenic part in particular is characteristic of a natural origin. A similar discriminating potential has been especially observed in linalool (Hanneguelle et al., 1992) and safranal (Remaud et al., 1993), for example.

The mean values of the relative isotope ratios $R_{2/1}$, $R_{3/2}$, and $R_{3/1}$ collected in Table 1 provide typical discriminating factors.

Table 2. Site-Specific Hydrogen Isotope Ratios Measured by SNIF-NMR and Total Isotopic Deviations of ^{13}C, ^{15}N, ^{34}S Measured by IRMS on Allyl Isothiocyanate Extracted from Mustard Seeds from Canada and India and Produced Chemically

sample no.	origin	δ^{13}C[a] (‰)	δ^{15}N[b] (‰)	δ^{34}S[c] (‰)	(D/H)I (ppm)	(D/H)II (ppm)	(D/H)III (ppm)	(D/H)IV (ppm)
50308	Canada	−28.3	9.2	−6.4	132.5	120.2	107.6	127.7
30280	Canada	−25.3	8.0	2.6	136.9	117.1	107.4	138.5
90238	Canada	−25.0	9.1	2.7	132.4	120.1	109.8	132.0
10125	Canada	−27.1	9.5	−8.3	139.0	120.5	114.8	137.9
10118	Canada	−28.0	9.5	−10.5	139.7	137.2	106.8	133.4
80349	Canada	−27.7	8.1	−3.8	137.5	121.4	111.8	131.5
60492	Canada	−28.1	8.1	−4.7	126.7	124.4	98.0	124.0
50310	Canada	−26.5	1.7	−17.1	nm[d]	nm	nm	nm
50307	Canada	−31.1	nm	nm	137.7	118.1	106.0	126.6
90345	Canada	−27.6	9.3	−7.4	138.4	120.2	110.8	136.0
	mean	−27.49	8.06	−5.88	135.64	122.13	108.11	131.96
	(9) SD	1.76	2.46	6.19	4.26	6.01	4.71	5.09
40363	synthetic	−26	0.2	−2.5	142.7	147.3	146.6	139.5
50309	synthetic	−26.3	−0.3	14.1	142.1	144.4	143.0	138.6
50226	synthetic	−28.2	−11.7	10.9	145.1	147.3	146.5	142.0
70051	synthetic	−29.5	−10.9	14.0	148.1	181.6	148.2	148.1
70050	synthetic	−30.3	−14.6	9.9	140.0	155.8	142.6	139.0
60491	synthetic	−29.6	−15.6	9.4	140.3	168.5	137.6	136.8
50008	synthetic	−28.7	−11.8	10.3	144.7	151.4	145.7	143.2
90005	synthetic	−31.1	−16.4	10.1	134	170.1	134.2	135.0
40180	synthetic	−29.5	−11.9	8.1	149.3	151.3	141.3	142.4
	mean	−28.80	−10.33	9.37	142.92	157.52	142.86	140.51
	(9) SD	1.72	6.14	4.88	4.63	12.84	4.60	3.90
10011	India	−28.2	16.5	11.3	143.9	130.0	105.7	135.5
50011	India	−27.8	14.6	11.6	145.0	117.7	114.6	145.2
50009	India	−28.3	19.0	6.8	144.7	120.7	113.2	145.5
50010	India	−27.6	17.0	12.4	142.8	123.1	113.4	135.9
50012	India	−26.7	14.4	11.2	148.5	120.5	115.1	150.9
	mean	−27.72	16.30	10.66	144.98	122.40	112.40	142.60
	(5) SD	0.64	1.89	2.21	2.14	4.66	3.83	6.70

[a] Vs PDB. [b] Vs air. [c] Vs CDT (see text). SD: standard deviation of the mean. [d] nm: not measured.

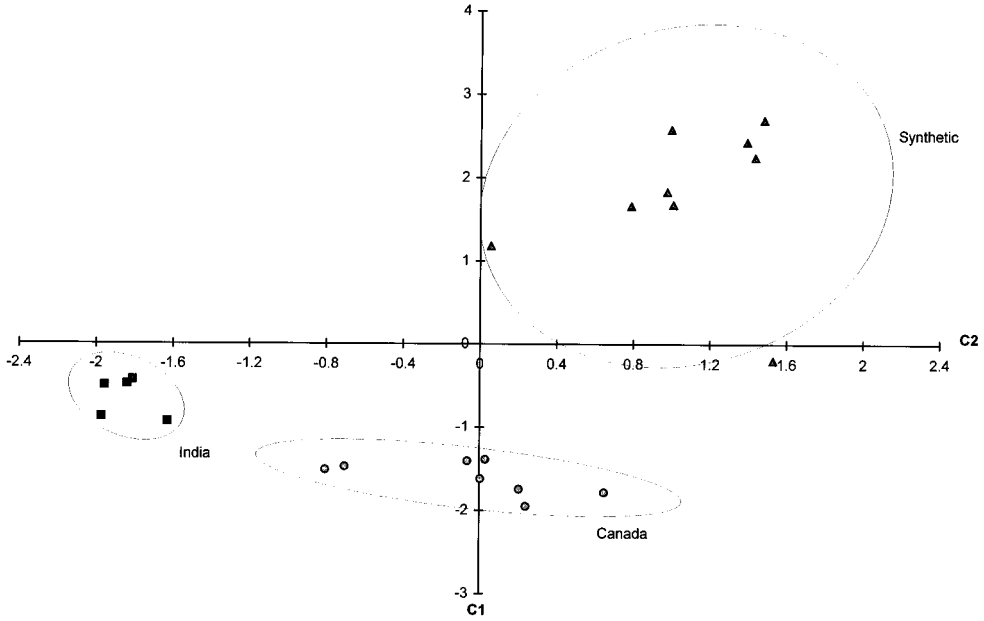

Figure 2. Principal component analysis performed on the analytical parameters $\delta^{13}C$, $\delta^{34}S$, $\delta^{15}N$, $R_{2/1}$, and $R_{3/1}$ of allyl isothiocyanates of natural origin (i.e. extracted from mustard seeds from India or Canada) or produced chemically. The results are represented in the plane of the two main components C_1, C_2. The ellipses represent the 99% bivariate confidence intervals.

(b) ^{13}C, ^{15}N and ^{34}S IRMS Analyses on Mustard Oil. The carbon 13 contents of allyl isothiocyanate from natural and synthetic origins (Table 2) are not significantly different. A noticeable overlap between the ^{13}C ranges is indeed observed since the mean value of the $\delta^{13}C$ deviation of allyl isothiocyanate ex-mustard seeds is equal to −27.6‰ with a standard deviation (SD) of 1.5‰ (15 samples) whereas that of synthetic allyl isothiocyanate is −28.8‰ with a SD value of 1.7‰ (nine samples).

The $\delta^{15}N$ results (Table 2) show that $\delta^{15}N$ is negative for the synthetic origin. We only observed one exception on one commercial sample (number 40363 in Table 2) for which we do not know the manufacturing process. It should be noted that the $\delta^{34}S$ deviation of this sample also seems atypical. Nevertheless some rules can be derived from the data presented in Table 2. For example, the nitrogen-15 and sulfur-34 contents of natural mustard oil from India are significantly higher than those of the Canadian origin. These isotopic parameters are particularly useful for identifying an Indian origin.

From a general point of view, a principal component analysis which projects the whole set of isotopic results on the plane of the two main components illustrates the performance of a multiisotopic approach (Figure 2). There is no overlap between the ellipses corresponding to the existence domains of the three different origins at the bivariate 99% confidence level.

(c) Detecting and Quantifying Adulterations. In most cases, the parameters $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ are not discriminating enough to allow an unambiguous distinction between natural and synthetic allyl isothiocyanates. For this purpose, the SNIF-NMR method is much more appropriate. In order to further estimate its hability to detect the adulteration of natural allyl isothiocyanate by its synthetic counterpart, mixtures containing known proportions of the two species have been investigated. The results have been mathematically processed by means of a statistical calculations involving a linear combination of the easily measured $R_{i/j}$ parameters, and a comparison of the composition derived from the isotopic results with the actual one is given in Table 3. Mixture A is composed of 22% of synthetic allyl isothiocyanate (no. 40363 in Table 2) and 78% of natural mustard oil (no. 80349 in Table 2). Mixture B is composed of 7% of no. 40363 and 93% of no. 80349. The agreement is satisfactory. More accurate statistical values could be obtained by including a larger number of reference samples in each group. However it may be concluded that SNIF-NMR is already able to easily detect adulteration of natural mustard oil with synthetic allyl isothiocyanate at a level as low as 10%. The samples (no. 80349 and no. 40363) used in the above example for producing mixture A and B are not a favorable case scenario from our database. A 7% adulteration is easily detected in this example. This underlines that in most cases adulteration will be detectable well below 10%.

Table 3. SNIF-NMR Analyses of Natural Mustard Oil Intentionally Spiked with Chemically Produced Allyl Isothiocyanate at 22% (Mixture A) and 7% (Mixture B)[a]

	actual values (%)		experimental values (%)	
sample	synthetic	natural	synthetic	natural
mixture A	22	78	17	83
mixture B	7	93	11	89

[a] The mixtures were made using sample no. 80349 as natural origin and sample no. 40363 as synthetic origin (see Table 2).

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