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Review

Isotopic Fractionation of Organic Compounds in Chromatography

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I. Introduction

During the past fifty years the development of synthetic methods to prepare and analytical methods to detect isotopically labeled organic compounds has made them exceedingly valuable tools for research. Stable isotopes such as ^{2}H, ^{13}C and ^{15}N have been used to elucidate biosynthetic schemes and reaction mechanisms, and radioisotopes such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, and ^{125}I have been exploited to clarify metabolic pathways, facilitate biological assays and deduce the structure of complex molecules like DNA.

After the synthetic preparation of an isotopically substituted organic compound it is a typical practice to corroborate isotopic product identity by performing cochromatography (demonstration of the same Rf or retention time) with an authentic nonlabeled standard in multiple chromatographic systems. This exercise is especially crucial for radioisotopes like tritium where the tiny mass of product involved often precludes alternative identity confirmation such as IR, NMR and the like. It is our experience that for the vast majority of cases such isotopically labeled compounds completely cochromatograph with authentic nonlabeled substances. However, for over forty years, some interesting and well documented examples have emerged for all types of chromatography where isotopically labeled compounds and authentic unlabeled standards simply do not cochromatograph. In fact in some cases dramatic baseline peak resolution can be achieved. This

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chromatographic phenomenon has been referred to as isotopic fractionation and was last reviewed by Klein (1) over thirty years ago. In this review the phrase "isotopic fractionation" refers only to chromatographic isotopic fractionation. Since Klein's review a partial discussion of the topic has appeared in several other limited publications (2-5).

The purpose of this discussion will be to review the documented literature instances of chromatographic fractionation for isotopically labeled organic compounds, especially the many intriguing examples that have been published since Klein's first review. It excludes a number of unpublished instances of the phenomenon for compounds confidential to us or those we have collaborated with. This summary was presented in part at a recent Philadelphia International Isotope Society (IIS) meeting (6) and the cited papers usually fall within two catagories. Some of them are totally devoted to an observation of isotopic fractionation often convincingly documenting it, exploring its scope, proposing an explanation or mechanism for its occurrence and placing it in context with other examples. However, many cited authors in diverse disciplines often disclose a single well characterized observation of isotopic fractionation as a curious side issue to the main body of their work. It is clear that some of these investigators were unaware of the phenomenon prior to their own observation and sometimes surprised by it. This review most likely captures all of the pertinent references on isotopic fractionation from 1955 to the end of 1997, but has excluded other articles whose observation or claim of the phenomenon was somewhat ambiguous. Articles were located not only by a painstaking and meticulous review of key chromatographic journals (tables of contents and indices) since 1955, but also by exhaustive cross reference checking of retrieved articles and citation searching of past papers to uncover later ones referencing them. The chromatographic techniques covered include gravity column, ion exchange, electrophoresis, paper, thin layer, gas and high pressure liquid chromatography. Although there are several reports of isotopic fractionation for the technique of countercurrent distribution (7-9), this topic will not be addressed here. Finally, the proposed factors causing chromatographic peak separation and its implications will be reviewed. Armed with this information, investigators in many life science areas using labeled

compounds will be better prepared to anticipate and interpret such results if they occur in their own laboratories.

II. Column Chromatography

Perhaps the first mention of isotopic fractionation for an organic compound in the chemical literature was by Van Dyken in the abstracts of a 1955 American Chemical Society meeting in Minneapolis (10) and later in a larger paper where the same data was incorporated and discussed (11). It was observed that on a silica gel column eluted with mixtures of butanol:chloroform, tritium labeled acids like formic acid manifested separation from their unlabeled standards. In most cases the tritiated acid eluted later than the unlabeled analogue as shown by specific activity (the measurement of radioactivity per unit of mass, often expressed as mCi/mmol) determinations of successive peak cuts.

Isotopic fractionation has been observed on column chromatography not only for such small molecules but also for much larger ones like the steroids [acetate-2-^{3}H] cholesterol acetate (12-14), [1,2-^{3}H] aldosterone (15-19), [^{3}H] 7-dehydrocholesterol (20), [1,2,6,7-^{3}H] testosterone (21), a tritiated ecdysone related compound (22) and lipids like [^{3}H] methyl linoleate (23). In many of these examples the coelution of analogues labeled separately with carbon-14 and tritium was examined by measuring the changing ratio of ^{14}C/^{3}H on successive peak cuts during chromatography. A great deal of effort was expended in ruling out impurities and other chromatographic artifacts as alternative explanations for the observations since many of these early reports were still met with relative disbelief (l). From these early studies several conclusions were already drawn about the phenomenon in the context of column chromatography. Tritium more often than carbon-14 appeared to cause isotopic fractionation and its presence almost always retarded compound elution on silica gel compared to the unlabeled substance. Also, the location of tritium labeling appeared to be a strong factor in the mechanism and extent of isotopic fractionation. Finally, it was appreciated that the degree of isotopic fractionation could be effected by column conditions.

Several examples of isotopic fractionation with stable isotope labeled organic compounds by column chromatography have been reported. A deuterium labeled

prostaglandin showed isotopic fractionation during column chromatography but its oxygen-18 analogue did not and use of the latter circumvented this "undesirable" property (24). Carotenoids labeled with deuterium could be separated from their unlabeled counterparts on activated magnesium oxide (25) and in this instance the deuterated compound migrated more slowly. Finally, several deuterated p-xylenes were separated by column chromatography from unlabeled analogues by cleverly taking advantage of a differential host/guest mechanism with tetrakis(4-methylpyridine)nickel (II) thiocyanate that had previously been employed to separate nonlabeled aromatic isomers (26). This appears to be the first report utilizing such an inclusion process to promote isotopic fractionation, but surprisingly the authors stopped short of suggesting what would seem to be a possible steric explanation (see Section IX).

III. Ion Exchange Chromatography

Although obviously related to column chromatography, ion exchange chromatography is unique enough to merit separate discussion. A very systematic study of isotopic fractionation as influenced by isotope location was the work of Piez and Eagle (27, 28) using ion exchange chromatography on several amino acids labeled with carbon-14. Elution of a Dowex 50 column with a pH gradient clearly demonstrated that several carbon-14 labeled amino acids migrated more slowly on the column than their unlabeled counterparts. It was evident from even this early work that the increased molecular weight of the radiolabeled compound alone could not satisfactorily explain the observed degree of isotopic fractionation, and it was also apparent that the position of the radiolabel in the amino acid had a marked effect on its mobility. In general, the greatest amount of isotopic fractionation was noted when carbon-14 was adjacent to the amine. Since this initial observation, other related instances of carbon-14 amino acid isotopic fractionation by ion exchange chromatography have been reported (29-33), discussing both the mechanism of the phenomenon and its implications. A separation was also described for [^{14}C] formic acid (34) via ion exchange chromatography on a strongly basic Dowex-2 resin. In this latter example the carbon-14 labeled compound was significantly enriched in the earlier eluent.

Several compounds labeled with tritium have been reported to show isotopic fractionation in this medium. [^{3}H] 2-Aminopurine eluted earlier than authentic unlabeled standard on a Dowex 1-X8 column (35). Special care was taken to exclude impurities as the cause of isotopic fractionation in this instance by paper chromatography analysis of fractions at the front and back of the radioactive peak. It was found that both the early and late eluting peak fractions were homogeneous and cochromatographed with authentic 2-aminopurine. Noting that the degree of isotopic fractionation was influenced by eluent pH, this report was also one of the first to implicate the influential role of tritium in modifying the polarity of an adjacent nitrogen atom. Several reports on the isotopic fractionation of tritium labeled amino acids by ion exchange chromatography have appeared (36, 37). It was observed that unlike carbon-14, which almost always resulted in a less mobile amino acid, the retention time of an amino acid labeled with tritium was very dependent on the radiolabel location. On a Dowex 1-X4 column, several carbohydrates including [6-^{3}H] maltose (38) and [6-^{3}H] D-glucose also exhibited isotopic fractionation (39). While there was only a slightly increased compound mobility in the former example, in the latter it was significant enough for the authors to speculate that with early fraction collection and recycling, very isotopically pure [6-^{3}H] D-glucose could be obtained. Also, the separation of tritium labeled folate derivatives from carbon-14 and unlabeled folate analogues (40) as well as the earlier elution of [^{3}H] ethylenediamine-ouabain (41) by ion exchange chromatography have been described.

A few reports have related the ion exchange isotopic fractionation of stable isotope containing organic compounds. [^{15}N] Urea was enriched in isotopic content by means of its isotopic fractionation on cation exchange chromatography (42) and several deuterated glucose isomers were separated by anion exchange chromatography (43). In this latter example using two CarboPac PA1 columns in series, baseline separation was realized for [1-^{2}H] D-glucose (earlier eluting) and unlabeled D-glucose. Also by this method almost baseline separation was achieved for [1-^{2}H] D-glucose (earlier eluting) from [2-^{2}H] D-glucose. The authors concluded that these results rule out mass or hydrophobicity as being operative in the separation. Rather, they proposed that the inductive effect of the [1-^{2}H] D-glucose

C-^{2}H bond reduces the adjacent C-O bond strength thereby increasing the adjacent O-H bond strength making it less acidic than in the case of the [2-^{2}H] D-glucose.

IV. Electrophoresis

Only a few examples of isotopic fractionation by means of electrophoresis have been described but several have been fairly striking. Giovanelli and coworkers reported the significant isotopic fractionation of [^{3}H] GSH sulfonic acid (44). With regard to stable isotopes, [^{2}H] dansylated methylamine separated with almost baseline resolution as a faster peak from unlabeled compound via micellar electrokinetic capillary chromatography (45, 46). In this study the addition of methanol to the mobile phase, use of long capillaries, high voltages and a stepped application of the voltage were key contributing factors to the successful separation observed.

Recently, Chiari described the complete separation of several deuterated compounds (such as [^{2}H] aniline, [^{2}H] benzoic acid and [^{2}H] pyridine) from unlabeled compounds by capillary zone electrophoresis (47). The mechanism suggested for this fractionation was that the electron donating inductive effect of ring deuterium (compared to hydrogen) altered the ionization constant of nearby functionality. Using this same technology, Terabe and coworkers were also able to completely resolve unlabeled benzoic acid (earliest eluting) from single (middle eluting) and double (last eluting) labeled [^{18}O] benzoic acid isotopomers (48). The authors optimized applied voltages, capillary tube lengths, buffer pH and electroosmotic velocities to achieve this stunning separation and proposed that this method was more efficient than HPLC to obtain the resolution of such closely related ionizable compounds.

V. Paper Chromatography

To date, instances of isotopic fractionation reported for paper chromatography have been few. Several deuterated amino acids and carbohydrates showed partial separation from authentic standards in this medium (49, 50). In both cases the deuterated compounds migrated more slowly than the unlabeled substances. Other examples include the separation of tritiated steroids

from cold or carbon-14 analogues during paper chromatography as demonstrated by either changing specific activity or $^{14}C/^{3}H$ peak ratios measured on the chromatograms (51-55). Several of the authors recognized and cautioned that the phenomenon must be taken into account for such studies as isotope dilution analysis of steroid secretion rates.

VI. Thin Layer Chromatography

The technique of thin layer chromatography (TLC) has only a few but certainly some of the most dramatic instances of isotopic fractionation yet observed. Perhaps the very first report of its occurrence on TLC was the example of [^{14}C] sodium formate, separated as a higher Rf spot on a basic silica gel system (56). TLC isotopic fractionation has also been reported for carbon-14 labeled dichlorobenzene isomers (57) as well as [1,2-^{3}H] cholesterol (58), [2,4-^{3}H] lithocholic acid (59), [^{3}H] Juvenile Hormone I (60), the calcium antagonist [^{3}H] VUF-4576 (61) and [^{2}H] methadone (62). Two studies on the TLC behavior of isotopically substituted analogues of imipramine have been described. Silica gel TLC of [benzene ring, N-methyl-^{2}H] imipramine in basic solvent systems such as methanol:ammonia (200:3) caused separation of it as a lower Rf spot from the higher Rf unlabeled imipramine (63). However, isotopic fractionation was not observed in acidic silica gel TLC systems. Also, no isotopic fractionation was observed for [benzene ring-^{2}H] imipramine, even in basic TLC systems. A photograph of the actual visualized TLC plates documented the separations observed in this study.

Our interest in tritiated radioligands for receptor binding studies prompted us also to study the TLC isotopic fractionation of [N-methyl-^{3}H] imipramine (64). Our results completely paralleled those just described since we too observed pronounced isotopic fractionation between (lower Rf) [N-methyl-^{3}H] imipramine and unlabeled imipramine in basic TLC systems but complete cochromatography between them in acidic TLC systems. Also, we saw no isotopic fractionation between [benzene ring-^{3}H] imipramine or [N-methyl-^{14}C] imipramine and unlabeled imipramine in any TLC system. In our case the measurement of TLC isotopic fractionation was exceptionally easy because of the ability after autoradiography to simply overlay the developed film upon the visualized TLC

plate. These results presented compelling evidence that the exclusive cause of isotopic fractionation for these compounds was deuterium or tritium substitution on the N-methyl groups. Therefore, although only a few examples of isotopic fractionation have been reported for TLC, it is important to be aware of its occurrence with the supportive role that this quick and convenient chromatographic method plays in the synthesis and characterization of labeled substances important to the life sciences.

VII. Gas Chromatography

With most of the aforementioned chromatography techniques, our discussion thus far of isotopic fractionation has been dominated by radioisotopes. Gas chromatography (GC) was a method where the property was first observed and widely studied for stable isotopes in general and deuterium in particular. Therefore it is only fitting to cover this isotope first. The subject was reviewed for selected small molecules almost thirty years ago by Van Hook (65).

The very first reported separation of any deuterated compound from its hydrogen analogue by GC would seem to be that of Wilzbach and Riesz (66). On a 4 meter didecyl phthalate column at 53°C with a helium flow rate of 45 mL/min, they were able to observe a partial separation of the earlier eluting perdeuterated cyclohexane from cyclohexane. Other workers using different GC conditions such as glass capillary columns (67), sandwiched capillary columns (68) and porous polymer beads (69) subsequently improved upon this same separation to almost baseline resolution. In these cases also, deuterated cyclohexane eluted faster than cyclohexane. Other reports of this separation (70, 71) as well as further examples of isotopic fractionation for other alkanes labeled with deuterium soon followed. They included 2,3-dimethylbutane (72), butane (73), ethane (74-78), ethylene dibromide (79) and alpha-1,2,3,4,5,6-hexachlorocyclohexane (80). The technique of recycle gas chromatography was exploited to enhance the separation factor in one of these investigations (73). Similar separations of a number of other deuterated alkanes have also been described (81-83). The isotopic fractionation of methane from its deuterated analogues has perhaps received the most attention (69, 73, 75, 77, 84-100). Some of these articles related the excellent separation of CH_4 and C^2H_4 with

the order of elution depending upon the GC conditions used. In one study (94) a marked difference in the separation between these isotopomers as a function of carrier gas on a Porapak S column was observed, and the degree of peak separation effected by each carrier gas was found to be in the order helium> argon> nitrogen> carbon dioxide. In several reports the reasonably clean separation of the four possible deuterated isotopomers of methane was achieved at low temperature. On a glass capillary column, the elution order was CH_4, $CH_3{}^2H$, $CH_2{}^2H_2$, CH^2H_3 and C^2H_4 (88). However, using graphon coated with 0.1% squalane, this elution order was reversed (77).

Besides saturated hydrocarbons, several other classes of deuterated compounds have demonstrated isotopic fractionation. Deuterium labeled olefins such as ethylene (69, 77, 101-112), propylene (101) and 2-butene (105) have been studied. In one of these reports (104) the use of a long silver nitrate-ethylene glycol column allowed nearly baseline separation of deuterated ethylenes differing by only a single deuterium. Additional modes of column complexation were operative in several other of these examples and in one of them (110) the use of a coordination compound, dicarbonylrhodium (1) 3-trifluoroacetylcamphorate, in squalane on a capillary column promoted the essentially complete resolution of all isotopomers of deuterated ethylene. The authors predicted that use of this technique would accomplish the separation of other deuterated olefins and that an enhanced separation could be anticipated for tritiated olefins. Other unsaturated deuterated hydrocarbons have also been found to exhibit isotopic fractionation (113).

Compounds labeled with deuterium such as benzene (69, 114-124), toluene (75, 116, 123-126) and xylenes (127-130) have also been examined. In one of these articles (115) the nature of the GC stationary phase was found to exert a profound effect on the degree of $C_6H_6/C_6{}^2H_6$ separation. Capillary columns coated with dinonyl phthalate, squalane and silicone oil afforded increasingly improved separations of the two compounds with perdeuterobenzene most often emerging as the earlier peak. In another study (116) almost complete separation of the isotopic pairs $C_6H_6/C_6{}^2H_6$ and $C_7H_8/C_7{}^2H_8$ was achieved <u>via</u> an open tubular thick layer graphitized carbon black column. One investigation (123) explored the relative influence that ring and side chain aliphatic deuteration would have in enhancing

peak separation and concluded that side chain labeling had more of an effect. The GC isotopic fractionation of other deuterated aromatics has been described (131, 132), and several reports about the separation of deuterated alkynes have also appeared. For deuterated acetylene (133) the order of elution on a Chromosorb P column was C_2H_2, C_2H^2H and $C_2{}^2H_2$. However, for the isotopomers of deuterated 2-butyne the order of elution on a capillary column was reversed with the most deuterated species eluting first (134).

More polar compounds labeled with deuterium have also demonstrated isotopic fractionation on GC. These include acetone (69, 116, 135-141), acetonitrile (77), chloroform (135, 141), ethanol (135-138), methanol (77, 135-137), pyridine (116, 136, 137) and 2-butanol (142). In ref. 141 the use of cyclodextrins as host inclusion compounds for isotopomer separation was reported for the first time. It was demonstrated that not only was the cavity size a major factor in isotopic fractionation but also the cavity environment and cyclodextrin ring subtituents were very influential. In the last study (142) the observation of the difference in NMR chemical shifts between deuterated and unlabeled alcohols with the NMR shift reagent $Eu(fod)_3$ inspired the authors to use it in effecting the GC separation. Further examples of GC isotopic fractionation for compounds labeled with deuterium include DMSO (143) and N-methylformamide and N, N-dimethyl-formamide (144). In the latter case it was noted that substitution with deuterium on the methyl group of the formamides lowered the GC retention time, but that its presence in the formal position increased retention time. GC isotopic fractionation of deuterated analogues has also been published for such ethers as tetrahydrofuran (145), where a cobalt (II) complex was employed, and di-2-butylether (146). The GC separations of several deuterated TMS carbohydrate derivatives (147-150) along with deuterated glucose and mannose (151) from unlabeled compounds have also been studied.

Other diverse deuterated compounds useful to life science investigators have manifested isotopic fractionation on GC including several examples of deuterated acids, amino acids or their TMS derivatives (152-156). It was found that deuterated TMS nucleoside analogues exhibited GC separation from their unlabeled counterparts (157). The deuterated analogues of uridine and cytidine were earlier

eluting but it was concluded that the degree of separation for each had more to do with structural considerations than label content. Other compounds labeled with deuterium such as TMS quinoxalinols (158), caffeine and its metabolites (159, 160), iproniazid (161), aminopyrine (162), isopropylantipyrine (163), verapamil (164), desipramine (165, 166) and fentanyl (167) have all displayed some degree of GC isotopic fractionation.

Deuterated lipids and leukotrienes have also been the subject of GC isotopic fractionation studies. Deuterated fatty acid esters (168, 169) and metabolites of deuterated linoleic and linolenic acids (170) and deuterium labeled acetonides of 6,9-octadecadiene (171) all displayed isotopic fractionation. Also, [$^2H_6$] valproic acid (172) and [$^2H_7$] 4-ketovalproic acid (173) separated on GC from the corresponding unlabeled compounds while a slight GC separation was noted between [$^2H_8$] LTB_4 and LTB_4 (174). Larger deuterated compounds like steroids have displayed GC isotopic fractionation from unlabeled counterparts too. [$^2H_5$] Testosterone (175) and [$^2H_5$] cholesterol (176) are such examples.

Although deuterated compounds have dominated the published instances of isotopic fractionation by GC, compounds labeled with tritium have also provided examples. By means of recycle GC, tritiated cyclobutane was separated as an earlier eluting peak from cyclobutane (73). Using a glass capillary column at low temperature the separation of CH_4, $CH_3{}^3H$, $CH_2{}^3H_2$, CH^3H_3 and C^3H_4 (increasing retention time) was reported (88). Curiously, the elution order was reversed using a charcoal column at low temperature (177). Other reports on the separation of tritiated methane isotopomers have appeared (178, 179) including one (180) which described the preparation and GC purification of C^3H_4 on a multi Curie scale. Other workers succeeded in the GC separation of several tritiated propane and butane isotopomers (181) and small reductions in the retention time of several compounds labeled with tritium including cyclohexane, cyclohexene, heptane and benzene compared to unlabeled compounds were noted (182). As with deuterated olefins, silver nitrate-ethylene glycol GC columns have promoted the separation of several tritiated olefins (183). A degree of GC isotopic fractionation has also been reported for larger tritiated molecules. Several tritiated lipid methyl esters have displayed some separation from their cold counterparts by GC (184, 185). Also,

monitoring the $^{14}C/^{3}H$ effluent ratio on the GC of certain tritiated steroids indicated that they were somewhat separated from their carbon-14 analogues (186).

Besides hydrogen isotopes, carbon isotopes have also demonstrated fractionation on GC. A small separation was noted for fatty acids labeled with carbon-14 (165). Also, a measure of GC separation has been observed for $^{12}CO/^{13}CO$ (187-189) and $^{12}CO_2/^{13}CO_2$ (190, 191), $^{12}CH_4/^{13}CH_4$ (192, 193) and $^{12}CF_4/^{13}CF_4$ (194). The investigation of isotopic fractionation for $^{12}CH_4/^{13}CH_4$ in ref. 193 is intriguing because it was prompted by the disparate ratios of these isotopomers in samples taken at various locations in a southern Italian gas field. The authors used Bentonite as a GC solid support to mimic what they propose was geological isotopic fractionation. Isotopic fractionation by GC for fatty acid methyl esters and other compounds labeled with carbon-13 has also been observed (195, 196). The GC isotopic fractionation of $^{11}CH_4$ from methane facilitated the improvement of its specific activity (197, 198), and a degree of GC isotopic fractionation was also reported for some nitrogen-15 labeled compounds (199).

VIII. High Pressure Liquid Chromatography

The most recent chromatographic technique to provide examples of isotopic fractionation is high pressure liquid chromatography (HPLC). In this area, reports of the phenomenon emerged almost simultaneously for both tritium and deuterium labeled compounds. With tritium, among the earliest examples reported were the reverse phase HPLC isotopic fractionation of the polycyclic aromatics [^{3}H] 7,12-dimethylbenz[a]anthracene, [^{3}H] benzo[a]pyrene and analogues (200-202). In these instances the tritiated material often eluted earlier than the cold standard. The HPLC of arachidonic acid metabolites on silver ion loaded columns also exhibited isotopic fractionation, with the separation of the earlier eluting unlabeled deuterated or carbon-14 compounds from the later eluting tritiated analogues (203-205). The authors proposed a unique and compelling explanation for the observation (see Section IX). Further reports concerning the HPLC isotopic fractionation of [^{3}H] arachidonic acid and other eicosanoids labeled with tritium followed (206-210), with several of the authors reflecting on the implications of the

phenomenon for biological assays. In the last reference Do and coworkers summarized over a decade of observations made in their laboratory on such separations for both normal and reverse phase HPLC with a variety of mobile phases. On reverse phase HPLC several tritiated steroids (55, 211) and on both normal and reverse phase HPLC a number of tritiated Vitamin D analogues displayed isotopic fractionation (212-216). Several of the authors noted that the degree of separation was influenced by tritium location. Worth (216) also speculated that the pronounced isotopic fractionation could be exploited to elevate the specific activity of tritiated Vitamin D metabolites.

Tritiated nucleosides and related compounds have also been studied. [^{3}H] Thymine (217) and [^{3}H] thymidine (218) analogues as well as [^{3}H] 2'-deoxyguanosine (219) were reported to separate on HPLC from unlabeled standards. The Kudelin group in St. Petersburg has employed isotopic fractionation on preparative HPLC to enhance the specific activity of tritiated nucleosides (220). In this account, specific activities were significantly increased with selective fraction collection of earlier eluting labeled compounds. [^{3}H] Dopamine (221) has also exhibited isotopic fractionation on reverse phase HPLC. This thorough study explored the influence of tritium location on separation and would appear to be one of the earliest accounts of the phenomenon for catecholamines. The drug substances [^{3}H] bepridil (222), [^{3}H] imipramine, [^{3}H] desipramine (223) and [^{3}H] N-0437 (224), and the macrocycle [^{3}H] tetrahydroechinocandin B (225) have also displayed HPLC peak fractionation. In the first two examples the authors noted a marked effect on peak separation in conjunction with the proximity of tritium to nitrogen. In contrast, the last example, initially disclosed at an October 1990 Merck hosted International Isotope Society meeting, is remarkable for the degree of separation provided by tritium in an extended alkyl chain location of the macrocycle remote from any heteroatom.

Several [N-methyl-^{3}H] radioligands have also afforded interesting cases of HPLC isotopic fractionation. On normal phase HPLC eluted with dichloromethane:methanol (98:2), we found that the elution order of (+-) mianserin analogues were unlabeled (+-)-mianserin, (+-)-[N-methyl-^{2}H] mianserin and finally (+-)-[N-methyl-^{3}H]-mianserin (226). The simultaneous use of both UV detection and liquid scintillation flow monitoring facilitated our measurement of isotopic

fractionation in this study. On reverse phase HPLC both [N-methyl-^{3}H] chlorpromazine (227) and [N-methyl-^{3}H] SKF (R) 83566 (228) demonstrated analogous HPLC separation. Interestingly, the retention time of the former radioligand was longer than the unlabeled standard while that of the latter was shorter than unlabeled standard on reverse phase HPLC. Also in the earlier study, no isotopic fractionation was reported for [benzene ring-^{3}H] chlorpromazine and the degree of isotopic fractionation for [N-methyl-^{3}H] chlorpromazine was significantly dependent upon pH.

For deuterated compounds the first example of HPLC isotopic fractionation recorded would seem to be the separation of long chain perdeutero carboxylic acids (earlier eluting) from unlableled standards on reverse phase by Thornton and Tanaka (229, 230). Baseline peak separation was noted for palmitic acid and perdeuteropalmitic acid prompting the authors to suggest the significance of their findings for possible preparative scale utility. Motivation for this study of the isotope effect during "hydrophobic binding" was its possible role as a model for similar interactions in biomembranes. [^{2}H] Methyl palmitate was separated from unlabeled standard employing a recycle technique on silver nitrate impregnated silica gel HPLC (231). This and similar methodology was later applied to other deuterated fatty acids (232, 233).

HPLC isotopic fractionation of monodeuterobenzene (234) as well as perdeuterobenzene (235-242), perdeuterotoluene (235, 238, 242, 243) and ethyl benzene (244) have been described. In most cases the deuterated compound eluted earlier than the unlabeled one. In one of these reports (239) it was concluded that deuterated water was superior to water for the HPLC separation of deuterated aromatics from unlabeled compounds, enhancing the separation by as much as 30%. Other deuterated aromatic compounds also have been investigated including [^{2}H] benzoic acid (245, 246). In the first study it was apparent that the position of deuterium labeling had a significant effect on the degree of fractionation observed, with ortho substitution being far less influential for separation than meta or para substitution. The author was inclined to propose a steric rather than electronic explanation for the observation. In the case of several aromatic aldehydes labeled

with deuterium on the aldehyde carbonyl, Yu and coworkers have described their HPLC separation from unlabeled compounds (247-249).

Other deuterated aromatic compounds reported to exhibit isotopic fractionation include [^{2}H] naphthalene 2-sulfonic acid (250), several deuterated polycyclic aromatics (251), [^{2}H] dopamine (252), [^{2}H] epinephrine and [^{2}H] norepinephrine (253) and [^{2}H] indole-3-acetic acid (254, 255). Several deuterated steroids (208, 256) and eicosanoids (204, 205, 257) have shown a degree of HPLC isotopic fractionation. Also the vitamin A metabolic precursor B-carotene, when deuterated, was found to separate with baseline resolution from unlabeled compound on reverse phase HPLC (258, 259), and in both reports the labeled compound eluted earlier. In the second study the authors showcased the results as an opportunity for other investigators without access to a mass spectrometer to study the dynamics of absorption and metabolism of [^{2}H] B-carotene in humans.

Deuterated drug type compounds have provided further examples of HPLC isotopic fractionation. These include [^{2}H] N-0437 glucuronide (224) several deuterated caffeines (260), [^{2}H] methadone (261), and some deuterated benzodiazepines and related compounds (236, 262-264). The effect of pH was noted as influential in some of these separations (262, 263). Also investigated were the tricyclic [N-methyl-^{2}H] imipramine (265) and the tetracyclic [^{2}H] mianserin (266, 267). In this latter example it was clear that only deuterium substitution on the piperazine ring of mianserin would promote the observed separation. The Organon group also investigated the related compound [^{2}H] Org GC-94 (268) which on normal phase eluted later than the unlabeled compound. In this case the more acidic the eluent, the better was the observed peak separation. Other examples of related HPLC isotopic fractionation include [^{2}H] carbamazepine (269), [^{2}H] CCNU analogues (270) and [^{2}H] roxatidine acetate metabolites (271) and [^{2}H] bepridil (222).

Various other deuterated compounds have also been reported to display isotopic fractionation including [^{2}H] tetrahydroechinocandin B (225) and several [^{2}H] gibberellins (272). In this latter case the labeled gibberellins eluted slightly ahead of the endogenous substances on reverse phase HPLC. Even the pigment chlorophyll (one of the earliest substances to be studied chromatographically by

Tswett) when deuterated, has demonstrated some striking reverse phase HPLC isotopic fractionation (273-275). Using the mobile phase water:methanol: acetonitrile:tetrahydrofuran (5:28:38:23) with a flow rate of 1 mL/min, Baweja reported that [^{2}H] chlorophyll a eluted two minutes ahead of the unlabeled compound (273). Reverse phase HPLC also accomplished the separation of [N-methyl-^{2}H] FTC-methylamine (276) as well as some perdeuterated diglucosyldiacylglycerols (277) from their unlabeled counterparts.

Other isotopes besides hydrogen have provided examples of HPLC fractionation. Both [^{14}C] trioleoylglycerol (278) and [^{14}C] leucine (279) appeared to separate from unlabeled standards. Further examples are [^{13}C] theophylline metabolites (280), [^{13}C] leucine (281), the antiviral compound [^{13}C] LY-217,896 (282), [^{13}C] decanal (283), some organic acids labeled with carbon-13 (284) and [^{13}C] methyl palmitate (285). Tanaka and coworkers have elegantly explored the isotopic fractionation of both nitrogen-15 and oxygen-18 labeled compounds. Although not completely resolved, significant HPLC separations of [^{15}N] aniline (286, 287), and [^{15}N] N-methylaniline and [^{15}N] N,N-dimethylaniline (287, 288). Others have reported similar HPLC isotopic fractionation of [^{15}N] chlorophyll (289). Tanaka has also reported the HPLC isotopic fractionation of the several isotopomers of [^{18}O] benzoic acid (290-292), [^{18}O] 4-chlorobenzoic acid (292) and [^{18}O] 4-nitrophenol (287, 292).

Perhaps the most intriguing aspect of isotopic fractionation for HPLC or any other chromatographic method has been very recently reported; namely, chromatographic separation based on the chirality conferred by isotopic content as initially reported by the Tanaka group (293-295). Indeed, in his first paper (293) Tanaka cited the fact that prior claims of diastereomeric separation based on isotopic influence have been challenged (296). In this demonstration of chiral isotopic fractionation, diastereomers of [phenyl-^{2}H] methyl 3-diphenylglycidate were separated on reverse phase HPLC as three peaks with the perdeuterated species eluting first. The center peak containing the two diastereomeric pairs created by single phenyl ring deuteration was separately caught and injected onto a chiral HPLC column whereby enantiomer separation was observed. Another equally compelling example of this phenomenon was reported recently by Pirkle (297),

disclosing that deuterated enantiomers of a pivalamide were separated in a chiral supercritical fluid HPLC experiment.

IX. Causes of Isotopic Fractionation

Although a great deal of effort has been expended in documenting various instances of isotopic fractionation, less time has been spent in explaining its mechanism. Indeed, after considering all the published work on the subject, it appears that a number of factors, varying in importance and each additive or subtractive in contributing to peak separation, may be operative. However, when considered collectively, these factors should adequately explain the elution order of substances differing only in isotopic content.

Mass:

In most cases the mass difference between isotopically labeled and unlabeled compounds alone has been dismissed as playing any significant role in isotopic fractionation. Isotopic substitution in many of the compounds reviewed here alter their molecular weight by only a few percent at best. However, the GC elution order of $^{12}CH_4$, $^{13}CH_4$, and $^{14}CH_4$ was consistent with their increasing mass differences (88).

Steric Considerations:

One would think that steric effects produced by the isotope itself would rarely be invoked as a cause of isotopic fractionation. However, a persuasive explanation advanced for the isotopic fractionation of some deuterated and tritiated compounds is a steric one; namely, the lowered zero point vibrational frequency and resulting decrease in the Van der Waals radius of the series H, ^2H, ^3H (298), and the increasingly shorter carbon-hydrogen isotope bond length in the series C-H, C-^2H and C-^3H. For instance: 1) On a silver nitrate-ethylene glycol GC column, the deuterated isotopomers of ethylene (101, 104) and propylene (101) can be separated. 2) On a silver nitrate-silica gel column, [9, 10, 12, 13-^3H] methyl linoleate migrated more slowly than the unlabeled standard (23). 3) On a silver nitrate impregnated normal HPLC column, [1-^{14}C] PGE_2 eluted faster than [5, 6, 8, 11, 12, 14, 15-^3H] PGE_2 (203). An explanation for all three of the foregoing separations is that the

decreasing C-H, C-^{2}H and C-^{3}H bond lengths allowed increasing interaction between the complexing silver ions of the solid support and the olefins of the isotopomers in question. It has been observed that the stability of silver ion-olefin complexes increased when the hydrogen atoms were replaced by deuterium (299). In this same study it was also noted that such silver ion-olefin complexes were more stable when deuterium was actually attached to the olefin bond rather than merely adjacent to it. Therefore, it can be argued that the progressive substitution of deuterium or tritium for hydrogen would retard to a greater degree the migration of an olefin on a silver ion loaded column. A similar explanation may be operative for the inclusion mechanism based separation of deuterated compounds in ref. 26 and the HPLC separation of deuterated benzoic acid isotopomers (245).

Vapor Pressure:

The vapor pressure of a labeled and unlabeled compound can differ (300-303). For instance, the vapor pressure of a deuterated compound is generally higher than that of the corresponding unlabeled compound (304). Certainly, this circumstance could be a factor influencing isotopic fractionation by GC.

Polarity:

The alteration of an atom's polarity by an adjacent isotope has been cited as the cause of some of the most intriguing instances of isotopic fractionation. From the work of Robertson (305, 306), it would seem that the substitution of deuterium or tritium on a carbon atom bearing a nitrogen would cause the nitrogen to be more basic than its unlabeled counterpart. During silica gel chromatography, the amine would more strongly deprotonate the weakly acidic Si-OH groups and thus retard the elution of the labeled compound relative to the unlabeled one. Obviously, this effect would be more pronounced in neutral or basic eluents, but in systems acidic enough to protonate all amines, such isotopic fractionation would normally be minimal. Polarity has also been suggested for the observed HPLC isotopic fractionation of aldehydes substituted at their carbonyl with deuterium, potentially altering their hydrophobic interaction with the reverse phase chromatographic system (247-249).

X. Implications

Having reviewed the many examples and probable causes of isotopic fractionation during the past several decades it is appropriate to briefly consider its implications. Clearly isotopic fractionation when considered over the entire experience of labeled compound chromatography is still a rare but certainly precedented observation and should only be invoked cautiously. If it is operative and not taken into account then significant errors can corrupt the measurements made with labeled compounds such as specific activity (33), various assays (209, 211) and mass spectrometry techniques (307). Awareness of it can prompt the use of an alternate label that does not display the phenomenon. Furthermore, isotopic fractionation has been exploited to facilitate various measurements including physical adsorption (91, 92), the dynamics of complexation (110), GC-MS analysis (154) and partition coefficient determination (241). It has also been used to substitute for more costly analyses like GC-MS (259) and provide models for such systems as biomembranes (229, 230).

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