SYNTHESIS AND STABILIZATION OF SELECTED HETEROCYCLIC AROMA COMPOUNDS

BY

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DISSERTATION

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ABSTRACT

Alkylpyrazines and alkanone substituted pyrrolines are heterocyclic compounds and are predominant odorants in various foods in which they provide pleasant nutty and roasty aroma characteristics. This study describes the development of technologies for the accurate and precise quantitation of flavor alkalpyrazines in foods and for the stabilization of some labile alkanone substituted pyrrolines for potential use in food flavoring applications.

The synthesis of deuterium labeled alkylpyrazines was successfully carried out through a novel and convenient procedure which was achieved by reacting labeled alkyl Grignard reagents with chloro-alkylpyrazines. Twelve isotopes, namely $[^2\text{H}_3]$-2-methylpyrazine, $[^2\text{H}_5]$-2-ethylpyrazine, $[^3\text{H}_3]$-2,3(or 6)-dimethylpyrazine, $[^2\text{H}_3]$-2,[$^2\text{H}_3$]-6-dimethylpyrazine, [$^2\text{H}_5$]-2,[$^2\text{H}_5$]-6-diethylpyrazine, $[^2\text{H}_5]$-2-ethyl-3(or 6)-methylpyrazine, 2,[$^2\text{H}_3$]-3,5-trimethylpyrazine, $[^2\text{H}_5]$-2-ethyl-3,6-dimethylpyrazine, [$^2\text{H}_5$]-2-ethyl-3,5-dimethylpyrazine, and 2,3-diethyl-$[^2\text{H}_3$]-5-methylpyrazine were prepared in good yields (57-100%) and high purities (86-98%). Use of synthesized isotopes for SIDA was evaluated using two distinctly different volatile extraction/isolation methods: solvent-assisted flavor evaporation (SAFE), and solid-phase micro-extraction (SPME). Results showed that 2,3-diethyl-5-methylpyrazine and 2-ethyl-3,5-dimethylpyrazine had the highest odor-active values among the 13 pyrazines quantified in commercial peanut butter, cocoa powder, and instant coffee. Besides, the average difference and relative standard deviation (%RSD) between SAFE and SPME was less than 10% (4.9%) and 3.6% respectively.

2-Acetyl-1-pyrroline (2AP) which provides a rice-like and roasty aroma was reported as the primary odorant in various foods. However, due to the highly
unstable nature of this compound, it is scarcely used commercially in flavor formulations. A novel and attractive method for stabilizing 2AP was successfully developed in this study. Coordination of 2AP to zinc ions (ZnI₂, ZnBr₂ or ZnCl₂) resulted in the formation in high yields of stable crystalline complexes. Heterocyclic nitrogen and carbonyl oxygen atoms were possible binding sites to the zinc ion as indicated by infrared spectroscopy. Stable complexes of other structural homologues of 2AP, including 6-acetyltetrahydropyridine(s), 2-propionyl-1-pyrroline, 2-acetyl-2-thiazoline, 2-acetylpyrazine, 2-acetylpiperidine, and 2-acetylthiazole could also be prepared by the same method. Stability studies showed that 2AP zinc iodide complex was stable at ambient temperature (only 6% reduction after 3 months of storage at 25°C). Meanwhile, the ATHP-ZnI₂ complex was similarly stable and showed over 88% retention after 2 months of storage.

The newly developed synthesis method for isotopically labeled alkylpyrazines is very important for other scientists who want to accurately and precisely quantitate pyrazines in foods. The discovery of the method for the complexation between 2AP and zinc ions may enable the practical use of the labile, yet powerful flavor compounds 2AP and its homologues as flavoring agents in foods. Among the zinc salts evaluated in this study, zinc chloride may be preferred for the complexation process since it has GRAS status. Temperature and moisture were found as primary factors that influence the stability of the 2AP-zinc complex. This information suggests that for practical use an appropriate package or encapsulation technology, such as dispersion in an edible oil or lipid, or wax encapsulation may be desired in order to protect the complex from moisture and interactions with other food components.
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# TABLE OF CONTENTS

## CHAPTER 1 INTRODUCTION ..................................................................................... 1

1.1 Heterocyclic derivatives ................................................................................. 1
1.2 Rationale and significance ............................................................................. 2
1.3 Study objectives .............................................................................................. 3
1.4 References ....................................................................................................... 5

## CHAPTER 2 LITERATURE REVIEW ................................................................. 7

2.1 Stable isotope dilution assay .......................................................................... 7
2.2 Pyrazines - formation and occurrence in foods ............................................. 9
2.3 Determination of alkylpyrazines in foods ...................................................... 10
2.4 Previous methods used for the synthesis of deuterium labeled pyrazines ... 11
2.5 2-Acetyl-1-pyrroline and 6-acetyltetrahydropyridine(s) - formation
    and occurrence in foods .................................................................................. 13
2.6 Synthesis of alkanone-heterocyclic compounds ........................................... 14
    2.6.1 Synthesis of 2-acetyl-1-pyrroline ........................................................... 14
    2.6.2 Synthesis of 6-acetyltetrahydropyridine(s). ......................................... 20
    2.6.3 Synthesis of 2-propionyl-1-pyrroline .................................................. 23
2.7 Stabilization of 2-acetyl-1-pyrroline and 6-acetyltetrahydropyridine(s). ... 24
2.8 Transition metals – organic compound complex formation ......................... 25
2.9 References ....................................................................................................... 27

## CHAPTER 3 CONVENIENT SYNTHESIS OF STABLE DEUTERIUM
    LABELED ALKYL PYRAZINES FOR USE IN STABLE
    ISOTOPE DILUTION ASSAYS ........................................................................... 35

3.1 Abstract ........................................................................................................... 35
3.2 Introduction ...................................................................................................... 36
3.3 Materials and methods ................................................................. 38
  3.3.1 Synthesis of 2-chloropyrazine ................................................. 39
  3.3.2 Synthesis of 2,6-dichloropyrazine .......................................... 40
  3.3.3 Synthesis of 2-chloro-3(or 6)-methylpyrazine .......................... 40
  3.3.4 Synthesis of 2-chloro-3,5-dimethylpyrazine ............................ 41
  3.3.5 Synthesis of 2,3-diethyl-5-chloropyrazine ............................. 42
  3.3.6 General procedure for the synthesis of alkylpyrazines .......... 43
  3.3.7 Compound identification ....................................................... 49
  3.3.8 Compound purities ............................................................... 49
  3.3.9 ^1H Nuclear magnetic resonance (NMR) ................................ 53
  3.3.10 Quantitation of selected pyrazines ...................................... 53
  3.3.11 Direct solvent extraction - solvent assisted flavor evaporation
        (DSE-SAFE) ............................................................................ 55
  3.3.12 Solid phase micro-extraction (SPME) .................................... 55
  3.3.13 Gas chromatography-mass spectrometry (GC-MS) ................ 56
3.4 Results and discussion ..................................................................... 56
  3.4.1 Direct Chlorination .................................................................. 57
  3.4.2 Chlorination of 2,6-dimethylpyrazine .................................... 57
  3.4.3 Chlorination of 2,3-diethylpyrazine ..................................... 59
  3.4.4 Deuterated alkylpyrazines .................................................... 60
  3.4.5 Quantitative analysis ............................................................ 62
3.5 References ......................................................................................... 64

CHAPTER 4 STABILIZATION OF THE POTENT ODORANT 2-ACETYL-1-
PYRROLINE AND ITS HOMOLOGUES BY COMPLEXATION
WITH ZINC HALIDES ........................................................................... 69
4.1 Abstract .......................................................................................... 69
4.2 Introduction ..................................................................................... 70
4.3 Materials and methods .................................................................... 72
CHAPTER 1

INTRODUCTION

1.1 Heterocyclic derivatives

Heterocyclic compounds such as alkylpyrazines and alkanone substituted pyrrolines impart pleasant roasty aroma notes to foods and have been reported as predominant odorants in many foods (Maga, 1982; Adams et al., 2006). Alkylpyrazines such as 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine are primary cocoa aroma compounds (Fraendorfer et al., 2006) and are often naturally present at low concentrations in many foods. In general, foods represent very complex matrices containing high levers of proteins, fats, and sugars/carbohydrates that interact with the flavor compounds (Suppavorasatit et al., 2012). This matrix interference makes quantitation of trace-level flavor compounds rather difficult. For this purpose, careful and precise quantitative methods such as stable isotope dilution assay (SIDA) are usually needed in order to obtain reliable and accurate results.

The alkanone-pyrroline compound 2-acetyl-1-pyrroline (2AP) is a significant flavor contributor in foods (Hofmann et al., 1998), and is regarded as the most powerful odorant in aromatic rice and bread (Buttery et al., 1983; Schieberle et al., 1987) due mainly to its very low odor threshold (0.1 µg/Kg in water) (Buttery et al., 1982). However, the highly unstable nature of 2AP has hindered its widespread commercial use. Pure 2AP is a clear and colorless liquid, but upon standing at room temperature it turns into black solid in just one hour. In aromatic rice, the amount of 2AP was found to decrease by 50% of its original content after storage for 3
months (Widjaja et al., 1996). The instability of 2AP has been postulated to occur via a polymeric reaction (Buttery et al., 1982).

1.2 Rationale and significance

To quantitate alkylpyrazines using SIDA, isotopically labeled alkylpyrazines are needed as internals standards. Several attempts have been made to synthesize labeled alkylpyrazines (Schieberle et al., 1987; Cerny et al., 1993; Chetschik et al., 2010). In those studies deuterium labeled organolithium reagents were reacted with alkylpyrazines for the preparation of the target isotopes. However, due to the fact that an alkylpyrazine is not an electrophilic substrate, this kind of synthesis usually results in very low yields, and there are other drawbacks such as low purity and complicated synthesis steps.

2AP is of commercial interest is due to its characteristic and unique pleasant aroma. However, this compound is not commercially available and is not used as a flavoring due to its great instability. Some effects have been made to increase the stability of 2AP. To our knowledge, microencapsulation of either synthesized or naturally isolated 2AP with β-cyclodextrin (β-CD), and/or maltodextrin, and /or starch is the only means studied thus far for the purpose of stabilizing the compound (Andreas et al., 2012; Apintanapong et al., 2003; Duby et al., 1996; Srinivas et al., 2006). However, these attempts suffer because of the following deficiencies: 1) stability of encapsulated 2AP is either not mentioned or is insufficiently studied; 2) low loadings of 2AP was used; and 3) 2AP is only stable at low temperature such as -20°C and 4°C.
1.3 Study objectives

The ultimate goals of this study were to develop technologies for the accurate and precise quantitation of flavor compounds in foods and for the stabilization of some liable flavor compounds for use as flavoring agents in foods. This led to the development of two hypotheses:

1) High yield synthesis of isotopically labeled alkylpyrazines can be accomplished by reaction of labeled alkyl Grignard reagents with chloro-alkylpyrazines. It was further hypothesized that synthesized isotopes would provide accurate and precise quantitative results in various foods by SIDA.

2) Transition metal ions complexes could be used for the stabilization of 2AP through the covalent bonding of the heterocyclic nitrogen and carbonyl oxygen on the 2AP molecule. Furthermore, the synthesized 2AP complex was hypothesized to maintain its stability during storage.

The following specific tasks were completed in this study:

1) Development of convenient synthesis of isotopically labeled selected alkylpyrazines, including methylpyrazine, dimethylpyrazines, ethylmethylpyrazines, ethyldimethylpyrazines, trimethylpyrazine, and diethylmethylpyrazine for use in SIDA. Various chloro-alkylpyrazines were synthesized beforehand for use as starting materials and then reacted with deuterated alkyl magnesium halides (Grignard reagents) to promote the efficient production of isotopically labeled alkylpyrazines. The chlorine groups (electrophile) on the pyrazine rings were selectively and efficiently attacked by the Grignard reagents resulting in almost 100% reaction yield. Synthesized labeled alkylpyrazines were used as internal
standards and two distinctly different extraction techniques were compared to assess the precision and accuracy of the SIDA method.

2) Another goal of this study was to increase the stability of 2AP and enable its potential use as a food flavoring. A novel stabilization method was applied which had never before been specifically used for the stabilization of a flavor compound. 2AP was coordinated as a ligand onto a zinc halide, thus forming a stable complex. Complexes of 2AP, as well its structural homologues such as 6-acetyltetrahydropyridine(s), 2-propionyl-1-pyrroline, 2-acetyl-2-thiazoline, 2-acetylpurazine, 2-acetylpyridine, and 2-acetylthiazole can be prepared with zinc chloride, zinc bromide, or zinc iodide in a similar manner. The stability of 2AP zinc iodide complex during storage and its chemical characteristic were studied in order to better understand the commercial potential this new technology.

This work is creative and original in that it is the first study to utilize corresponding chloroalkylpyrazines for the efficient synthesis of isotopically labeled flavor-associated alkylpyrazines. In addition, this study is the first to apply a novel procedure to stabilize 2AP by coordination to zinc ions to form highly stable complexes. In addition, zinc-complexation has never been used intentionally for the stabilization of any flavoring compound. Other structural homologues of 2AP such as 6-acetyltetrahydropyridine(s) and 2-propionyl-1-pyrroline, which are all unstable like 2AP, could also be stabilized by the same procedure. The stabilization mechanism was postulated to be due to a covalent linkage between the zinc ion and ring nitrogen and carbonyl oxygen atoms of the 2AP molecule.
1.4 References


CHAPTER 2
LITERATURE REVIEW

2.1 Stable isotope dilution assay

Stable isotope dilution assay (SIDA) is a state-of-the-art method for quantitative analysis, enabling both high precision and accuracy. The first published use of SIDA on a food material was in 1966, in which D-glucose containing seven deuterium atoms was used as an isotope tracer for the determination of glucose (Sweeley et al., 1996). SIDA, in which a stable isotope labeled (typically deuterium or carbon-13 is used for labeling) analogue of an unlabeled analyte is used as the internal standard during quantitative analysis. The stable isotope could be considered as the ultimate internal standard because of its great similarity in both physical and chemical properties to its unlabeled counterpart, the target analyte. After spiking and equilibration of a known amount of the stable isotope in the sample, the ratio of labeled and unlabeled compounds is maintained throughout extraction, workup and analysis steps (figure 2.1). Consequently, the two isotopologues, which differ in mass, are detected and differentiated by a mass spectrometer (MS) and the mass ion ratio of the two is used to determine the abundance of the target analyte in the initial sample.

SIDA has been applied for detection and quantitation of food contaminants by the U.S. Food and Drug Administration (FDA) (USFDA, 2002), and utilized in the investigation of food flavor (Munch et al., 1998), and for environmental hazards and pesticide residues (Cai et al., 2004). Despite its many advantages, SIDA is not widely used today due to the high cost associated with the purchase and/or synthesis of the isotope labeled standards. For example, the syntheses of deuterium
labeled pyrazines have been challenging because of low yields and low purities based on previous literature. Chapter 3 of this dissertation describes an improved method for the synthesis of deuterium labeled alkylpyrazines to be used as internal standards for quantitative analysis (i.e. SIDA) of selected important alkylpyrazines in commercial peanut butter, cocoa powder, and instant coffee.

Figure 2.1 Concept of SIDA, where the isotopologue ratio remains stable throughout extraction and analysis
2.2 Pyrazines - formation and occurrence in foods

Pyrazines belong to a class of heterocyclic nitrogen-containing compounds which contain four carbon and two nitrogen atoms in a ring skeleton (figure 2.2). Various combinations of side groups on positions 2, 3, 5 or 6 provide for a variety of substituted pyrazines having various odor characteristics (e.g., nutty, earthy and roasty) and different odor detection thresholds (Fors, 1983).

![Figure 2.2 Pyrazine. (R1-4 = hydrogen, alkyl group, alkoxy group, alkanone group, etc.)](image)

The earliest reports of pyrazines in food consist of patent documents from 1926 and 1928 which described methods for the preparation of natural coffee essential oil (Reichstein et a., 1926) and artificial coffee oil in which pyrazines were added (Reichstein et al., 1928). Alkylpyrazines can be formed from amino acids and reducing sugars via the Maillard reaction and Strecker degradation, which occur in heated foods, especially those which are toasted or roasted in their preparation (Hwang et al., 1995). Koehler and coworkers demonstrated the formation of amino carbonyls from the condensation of amino acid bound nitrogen with the carbonyl of a sugar, which in turn condense to form pyrazines (Koehler et
Pyrazines have been identified in various heat processed food products including nuts, soy, rice, egg, cocoa, cheese, bread, potato, vegetable, meat and seafood products and others (Maga, 1982). Therefore, pyrazines contribute, at least partially, to the overall flavor of nearly all heated foods. In particular, they have been reported as character-impact odorants in blanched oven roasted peanuts (Schirack et al., 2006), roasted beef (Cerny et al., 1993), French fries (Wagner et al., 1998) and coffee (Blank et al., 1992).

2.3 Determination of alkylpyrazines in foods

Pyrazines, as in the case of most volatile compounds, can be isolated from food systems by various methods. Steam distillation (atmospheric or vacuum) combined with solvent extraction has been commonly used (Herent et al., 1998; Mussinan et al., 1973). A main limitation of distillation, especially when conducted under atmospheric conditions, is the potential for artifact formation (Koehler et al., 1969). This may be overcome by a mild and exhaustive distillation technique called solvent-assisted flavor evaporation (SAFE) (Engel et al., 1999). Other methods used without distillation include supercritical fluid extraction (Leunissen et al., 1996) and solvent free techniques such as headspace analysis (Warner et al., 1996) and solid-phase microextraction (SPME) (Baker et al., 2003).

In the identification of pyrazines, capillary gas chromatography (GC) coupling with a mass spectrometer has been the preferred method. The use of a nitrogen specific GC detector (e.g., nitrogen-phosphorus detector), has been used for the selective analysis of pyrazines (Herent et al., 1998). Other GC detectors used for the analysis of pyrazines include flame ionization and thermal conductivity
detectors (Herent et al., 1998; Manson et al., 1966). The application of gas chromatography-olfactometry (GCO) techniques has enabled the identification of high (odor) potency pyrazines which exist in trace concentrations. For example, the roasty/nutty smelling compounds 2-ethyl-3,5-dimethylpyrazine and 2-ethyl-3,6-dimethylpyrazine, with low odor detection thresholds of 2.2 ng/g and 57 ng/g in oil (Warner et al., 1996), respectively, were identified as potent odorants in peanut based on GCO and molecular sensory analysis (Schirack et al., 2006). This is in contrast to previous reports which indicated the more highly abundant methylpyrazine and 2,5-dimethylpyrazine were important aroma contributors in roasted peanut (Baker et al., 2003); however, these compounds have low odor-activity values (OAVs) because of their relatively high odor detection thresholds of 27 µg/g (methylpyrazine) and 17 µg/g (2,5-dimethylpyrazine) in oil (Koehler et al., 1971).

2.4 Previous methods used for the synthesis of deuterium labeled pyrazines

To our knowledge the first use of SIDA for the quantification of pyrazines was done by Schieberle and Grosch who determined the concentrations of acetylpyrazine and 2-methyl-3-ethylpyrazine in wheat and rye bread crusts (Schieberle et al., 1987). In that study, deuterated acetylpyrazine was prepared from pyrazinamide and magnesium $[^2\text{H}_3]$-methyl iodide. Labeled 2-methyl-3-ethylpyrazine was synthesized by the condensation of 2,3-pentanediol and $[^2\text{H}_4]$-ethylenediamine. This was followed by fractionation using column chromatography (aluminum oxide) and further purification by thin layer chromatography (TLC), which resulted in isolation of very low quantities of target compounds. In a later
study by the same research group, two character-impact odorants 2,3-diethyl-5-methylpyrazine and 2-ethyl-3,5-dimethylpyrazine were quantified in roasted beef by SIDA (Cerny et al., 1993). The deuterated isotopologues of the above compounds were synthesized by treating 2,3-diethylpyrazine or 2,6-dimethylpyrazine, respectively, with \( ^2\text{H}_3 \)-alkyl lithium. The labeled compounds were isolated by TLC and then purified by high performance liquid chromatography (HPLC). Three alkoxy pyrazines, specifically 2-isobutyl-3-\( ^2\text{H}_3 \)-methoxypyrazine, 2-isopropyl-3-\( ^2\text{H}_3 \)-methoxypyrazine and 2-secbutyl-\( ^2\text{H}_3 \)-methoxypyrazine were synthesized by treating their unlabeled counterparts with hydrogen chloride to form the corresponding 2-alkyl-3-hydroxy pyrazines. The hydroxy derivatives were then treated with gaseous \( ^2\text{H}_2 \)-diazomethane in \( ^2\text{H}_3 \)-methanol. Reaction mixtures were subjected to silica gel chromatography to obtain purified labeled compounds (Semmelroch et al., 1996; Masanetz et al., 1998). Czerny and others prepared \( ^2\text{H}_3 \)-2-methoxy-3,5-dimethylpyrazine by refluxing 2,6-dimethylpyrazine with chlorine in carbon tetrachloride followed by treatment of the residue with sodium \( ^2\text{H}_3 \)-methoxide in \( ^2\text{H}_3 \)-methanol. TLC and preparative GC were applied for purification (Czerny et al., 2000). Other deuterated alkylpyrazines, namely methylpyrazine, dimethylpyrazine and trimethylpyrazine have been prepared by nucleophilic addition via the organolithium reagent \( ^2\text{H}_3 \)-methyl lithium with either pyrazine, methylpyrazine or 2,5-dimethylpyrazine, respectively (Chetschik et al., 2010). The target compounds were isolated in low yields by TLC.
2.5 2-Acetyl-1-pyrroline and 6-acetyltetrahydropyridine(s) - formation and occurrence in foods

2-acetyl-1-pyrroline (2AP) was first identified in cooked rice (Buttery et al., 1982) and was indicated as the most important flavor component among all identified rice volatiles (Buttery et al., 1983). Aromatic rice contains high levels of 2AP which is not formed either during postharvest processing or cooking, but instead is formed in the rice plants while they are growing in the fields (Yoshihashi et al., 2005). In commercial varieties of aromatic rice the concentrations on 2AP rage from 19 – 999 ng/g (Bergman, et al., 2000). 2AP also is found in Pandan leaves. In India, pandan leaves are traditionally used in the cooking of non-aromatic rice varieties to impart an aromatic rice-like quality. 2AP was found up to 7.163 mg/kg in supercritical carbon dioxide extracts from Pandan leaves, which contain about 10 times greater concentration of 2AP than aromatic rice (Bhattacharjee et al., 2005). Bread flower (Vallaris glabra Ktze) is another biological source of 2AP in which the amount of 2AP was found to be 26.12 mg/kg and 3.36 mg/kg in the dried and fresh flowers, respectively (Wongpornchai et al., 2003).

The Maillard-type reaction between the amino acid proline and reducing carbohydrates is well-known to generate roasty, popcorn-like odorants upon thermal processing. It is well accepted that 1-pyrroline formed form proline via the Strecker degradation is the key intermediate in the formation of both food odorants 2AP and 6-acetyltetrahydropyridine(s) (ATHP). In a model solution, proline was boiled with 2-oxopropanal for 2 h resulting in the formation of 2AP and ATHP (Schieberle, 1990). Replacement of 2-oxopropanal with fructose enhanced the
formation of ATHP but no 2AP was formed. ATHP is preferentially formed in popcorn over 2AP, but in bread crust 2AP is the dominant referentially generated in bread crust than ATHP. That could depend significantly on the carbohydrate cleavage products present in foods. If high amounts of 2-oxopropanal are present, 2AP will be formed, whereas in the presence of its reduction product hydroxy-2-propanone, formation of ATHP is favored (Hofmann et al., 1998).

Because 2AP and ATHP are generated during thermal treatment of foods these odorants can be found in almost all cooked foods. 2AP is a predominate odorant in corn tortillas (Karahadian et al., 1993), extruded potato snacks (Majcher et al., 2005), cooked tail meat of American lobster (Lee et al., 2001), nonfat dry milk (Karagul-Yuceer et al., 2001), and sweet corn (Buttery et al., 1994). 2AP, though mostly present in low concentrations in foods, plays a very important role due to its low odor threshold, 0.1 parts-per-billion in water (Buttery et al., 1982). Numerous studies have demonstrated the importance of 2AP in a variety of food products, such as Italian hazelnuts (Burdack-Freitag et al., 2010), pan-roasted peanut meal (Chetschik et al., 2008), pan-fired green tea (Kumazawa et al., 2002), thermally treated yeast extracts (Munch et al., 1998), popcorn (Schieberle, 1991), cooked rice (Buttery et al., 1982), and pandan leaves (Buttery et al., 1983).

2.6 Synthesis of alkanone-heterocyclic compounds

2.6.1 Synthesis of 2-acetyl-1-pyrroline

Various methods have been reported in the literature for the synthesis of 2-acetyl-1-pyrroline (2AP). The first synthesis started from the hydrogenation process that reduced 2-acetyl-1-pyrrole into 2-(1-hydroxyethyl)pyrrolidine via
rhodium on alumina catalyst and hydrogen. Because nitrogen heterocyclic compounds deactivate hydrogenation catalysts, a large quantity of expensive rhodium was necessary to complete the hydrogenation. A subsequent oxidation step via addition of silver carbonate on celite in benzene gave the product 2AP (Scheme 2.1). This procedure yielded less than 10% of the target compound and several by-products were formed. For this reason the reaction mixture had to be separated by preparative GC in order to obtain pure 2AP (Buttery et al., 1982; 1983).

Scheme 2.1

A large-scale method was reported by De Kimpe et al. (1993) for the synthesis of 2AP. This technique involved the oxidation of methylprolinate to 2-(methoxycarbonyl)-1-pyrroline which was then reacted with methyl magnesium iodide (Grignard reaction) (Scheme 2.2). The reaction did not go completion and resulted in only a 45% ~ 83% yield of 2AP. The compound 2-(1-hydroxy-1-methylethyl)-1-pyrroline was produced (8-39%) as a side product because treating an ester [i.e., 2-(methoxycarbonyl)-pyrroline] with the Grignard reagent is generally problematic. A more sophisticated procedure for this Grignard reaction was required or inevitably the accompaniment of a tertiary alcohol could ruin the reaction. An alternative solution was developed, which involved treatment of cyanide functional group instead of ester with the Grignard reagent (Scheme 2.3).
The preparation of 2-cyano-1-pyrroline started from the oxidation of pyrrolidine to 1-pyrroline which spontaneously underwent trimerization to tripyrrolidine. Tripyrrolidine was subsequently hydrocyanated and then oxidized to form 2-cyano-1-pyrroline, which was treated with methyl magnesium iodide to afford 2AP with minimal side products (De Kimpe et al., 1993).

**Scheme 2.2**

![Scheme 2.2](image)

**Scheme 2.3**

![Scheme 2.3](image)

Some other synthesis methods employed carbonyl-protected analogues to improve the yield because of the instability of 2AP (Scheme 2.4). These included a process which involved reacting ethoxyvinylthium with N-trimethylsilylpyrrolidinone in tetrohydrofuran at -40°C, with subsequent hydrolysis via ammonium chloride to obtain 2-(ethoxy-1-ethenyl)-1-pyrroline. Hydrolysis with strong acid (e.g 10.5N HCl ) was used to convert this intermediate to 2AP after neutralization (Duby et al., 1993; 1994).
Another process which involved the use of a carbonyl-protected process involved the α-deprotonation of diimine via lithium diisopropylamide (LDA), and α-alkylation with a stabase adducts (tetramethyldisilylazabromoethane) derivative to form 2AP along with a major side product after acidic and basic workup (Scheme 2.5). (De Kimpe et al., 1996).

Pure 2AP was produced by a synthesis method which utilized the high substrate selectivity of immobilized penicillin acylase (PGA) as the catalyst in the final step (Scheme 2.6). The synthesis began with the N-group protection of 1-amionhex-4-yne with phenylacetyl chloride. This compound was subsequently oxidized by treatment with ozone to afford 1-[N-(phenylacetyl)amion]-4,5-dioxohexane. Treatment of the later compound with PGA produced amino
diketones which spontaneously underwent ring closure to form 2AP in 80% yield and in pure form (Favino et al., 1996).

**Scheme 2.6**

Hofmann et al. (1998) reported a four-step synthesis of 2AP starting from the conversion of N-tert-butoxycarbonyl protected proline into the thioester, N-(tert-butozycarbonyl) - 2 - [ ( 2-pyridylthio ) carbonyl ] pyrrolidine (scheme 2.7). Subsequent Grignard reaction of the thioester with methyl magnesium bromide and deprotection via trifluoroacetic acid yielded 2-acetylpyrrolidine trifluoroacetate, which could be used as a stable intermediate for quickly generating 2AP via an oxidation process in water and pure oxygen.

**Scheme 2.7**

A short synthesis strategy was developed which involved the conversion of an N-BOC protected 2-pyrrolidinone to 1-ethoxy-1-lithioethene (produced by reaction of ethyl vinyl ether and tert-butyllithium). Treatment with 10% p-toluenesulfonic acid produced an intermediate N-BOC-2-acetyl-2-pyrroline with
corn-like smell which could be deprotected via trifluoroacetic acid (TFA) treatment to produce 2AP in 22% yield with the tautomer of 2AP as the major contaminant (Scheme 2.8) (Harrison et al., 2005).

Scheme 2.8

A regioselective oxidation on the side chain of 2-ethyl-1-pyrroline provided an efficient procedure for the preparation of 2AP (Scheme 2.9). The process started form the addition of ethyl group into N-BOC-pyrrolidinone via reaction with ethyl magnesium bromide. Subsequent deprotection with TFA removed the BOC with rearrangement of the pyrrolidine ring into a pyrroline ring. Selective site oxidation was conducted using selenium dioxide and tert-butyl hydroperoxide. The desired compound was obtained after workup, with an overall yield of 20~30% and purity of 98% (Fuganti et al., 2007).

Scheme 2.9

In a recent study, the synthesis started from L-glutamic acid and acetic anhydride to form N,5-diacetylpyrrolidin-2-one. This compound was deactylated using sodium carbonate and then reduced via lithium aluminium hydride to give 2-
(1-hydroxyethyl)pyrrolidine (Scheme 2.10), which was oxidized via silver carbonate to produced 2% 2AP (Maraval et al., 2010).

Scheme 2.10

2.6.2 Synthesis of 6-acetyl tetrahydropyridine(s)

The fresh bread flavor compound 6-acetyl-1,2,3,4-tetrahydropyridine (and its tautomer 6-acetyl-2,3,4,5-tetrahydropyridine, ATHP) was discovered earlier than 2AP. The synthesis of this compound could be accomplished by hydrogenation of 2-acetylpyridine over a rhodium on alumina catalyst, with subsequent oxidation in benzene with silver carbonate suspended in celite to obtain desired product (Scheme 2.11). Consequently, the imine 6-acetyl-2,3,4,5-tetrahydropyridine was the initial product. However, the enamine 6-acetyl-1,2,3,4-tetrahydropyridine was more stable and increased with time during the reaction (Büchi et al., 1971).

Scheme 2.11
Another synthesis procedure for ATHP started from 6-cyano-2,3,4,5-tetrahydropyridine prepared via the oxidation of piperidine to tripiperideine, which was hydrocyanated into 2-cyanopiperidine. The further oxidation driven by t-butyl hypochloride (t-BuOCl) and triethylamine (TEA) mediated dehydrochlorination yielded 6-cyano-2,3,4,5-tetrahydropyridine and a corresponding enamine 6-cyano-1,2,3,4-tetrahydropyridine. Subsequent Grignard reaction offered two isomers: an imine and an enamine in a ratio of 4:1 in the freshly prepared mixture, but the ratio gradually changed to 1:2 upon standing (Scheme 2.12) (De Kimpe et al., 1993).

Scheme 2.12

A N-protected proline pyridyl thioester was also used for the preparation of ATHP (Scheme 2.13). It is well-known that treating esters with Grignard reagents only yields alcohols, not ketones. The author introduced pyridyl thioester for Grignard reagent and subsequently yielded a ketone in 71%. Synthesis began with the formation of a pyridyl thioester. N-tert-butoxycarbonyl piperolic acid was treated with dipyridyl disulfide to form N-(tert-butoxycarbonyl)-2-[(2-pyridylthio)carbonyl]piperidine. Treatment of this compound with methyl magnesium iodide, and subsequent deprotection yielded ATHP in an overall yield of 61% (Hofmann et al., 1998).
Another strategy for synthesis of ATHP involved reacting N-BOC-2-piperidone with 1-ethoxy-1-lithioethene to produce a ketone which was subsequently treated with p-toluenesulfonic acid and then trifluoroacetic acid to form the target compound in 66% yield (Scheme 2.14) (Harrison et al., 2005).

A general method was reported for the synthesis of alkanone heterocyclic compounds including both 2AP and ATHP. The strategy involved the regioselective oxidation of the heterocyclic side chain. The method was already described for the synthesis of 2AP (Scheme 9). Replacing the starting material with 6-ethyl-2,3,4,5-tetrahydropyridine, the same synthesis procedure could be used for the preparation of ATHP with an overall yield of 20~30% (Scheme 2.15) (Fuganti et al., 2007).
2.6.3 Synthesis of 2-propionyl-1-pyrroline

The aforementioned method of Favino et al. (1996) method for the synthesis of 2AP could be adapted to for the synthesis of 2PP because of its structural similarity to 2AP. Replacement of 1-[(N-(phenylacetyl)amion]-4,5-dioxohexane which was used for the preparation of 2AP with 1-[(N-(phenylacetyl)amion]-4,5-dioxoheptane and use of a PGA catalyst yield 2PP (Scheme 2.16) (Favino et al., 1996).

The versatile method of Fuganti et al. (2007) could also be used for the oxidation of 2-propyl-1-pyrroline via selenium dioxide and tert-butyl hydroperoxide in a scheme which is analogous to the aforementioned syntheses of 2AP and ATHP (Scheme 2.17).
2.7 Stabilization of 2-acetyl-1-pyrroline and 6-acetyltetrahydropyridine(s)

The unstable/labile compound 2AP is an organic base and can be prepared as stable solid salt by reaction with acids. Buttery et al. (1985) prepared hydrochloride salt of 2AP by adding 36.5mg HCl to 111mg of 2AP in 200 mL of water. The salt which formed was obtained after evaporation, but there was no stability data provided (Buttery et al., 1985).

In another study, synthesized 2AP in water was added to β-cyclodextrin (β-CD) or maltodextrin as supporting materials. The solution was freeze dried to obtain a white powder in which 2AP was encapsulated in the supporting materials. 2AP at a concentration of 1% in either a maltodextrin or β-CD matrix was stable at -20°C for 110 days, decomposing by only 13% and 0%, respectively. However, 2AP at a concentration of 1% completely decomposed at 20°C after 50 days and 110 days in the maltodextrin and β-CD matrices, respectively. 2AP in high concentration of 10% in β-CD was unstable and decomposed by 91% at 20°C after 13 days (Duby et al., 1996).

Other supporting materials such as gum acacia or starch were also used for the stabilization of 2AP. The processes included addition of an ethanolic solution of
2AP into gum acacia or/and starch aqueous solutions containing Tween 60 as emulsifier. The solution was subjected to vacuum shelf drying (30-60°C) or spray drying (140°C) to obtained dispersible dry powders. The ratio of 2AP to supporting material was 1/2000. However, the stability of encapsulated 2AP was not mentioned (Srinivas et al., 2006).

Another approach started from crude extract of Pandan leaves which was mixed with β-CD at concentrations from 0.5 to 3 ppm. Some free amino acid and 3-methyl-2(5H)-furanone was added into the mixture for enhancing the organoleptic properties. Subsequent spray drying (160°C) gave a powder. There was no stability data reported (Andreas et al., 2012).

Lower concentrations appear to benefit the stability of 2AP encapsulated in support materials because this limits the self-polymerization reaction/degradation of 2AP. In different combinations of gum acacia and maltodextrin, 2AP at a very low concentration of 0.003% showed about 70% retention after 72 days of storage at ambient temperature (Apintanapong et al., 2003).

For the stabilization of ATHP only one published study was found. Proline and dihydroacetone were heated at 80-100°C in the present of bisulfite to generate crude ATHP which was converted into relatively stable bisulfite complex (Hunter et al., 1971 and 1973).

### 2.8 Transition metals – organic compound complex formation

The binding of transition metal ions to aromatic ligands is of interest as a fundamental phenomenon of metal-ligand interaction. Some aromatic compounds can offer multiple binding functionalities, such as an N-donor moiety (like an
amino group) or a keto-group (Dunbar et al., 2006). Common examples of this are iron in hemoglobin and also zinc finger proteins. Complexes such as acetophenone with Fe+, Co+, and Ni+ were found to provide a more attractive binding site of carbonyl oxygen-metal binding than the benzene ring for this metal ion (Dunbar et al., 2006). The nitrogen molecule in a pyridine ring aids in the self-assembly with zinc metal salt to form a complex, and the metal-nitrogen bond creates a chemical, so called π-stacked, linkage (Dias et al., 2004). The tendency of the metal-ligands coordination can be applied for the protection of unstable compounds such as the stabilization of 1-pyrroline as a stable crystalline complex with zinc ion, which can be regenerated by treatment of the complex with ammonia (Baxter et al., 1991).

2AP along with other selected alkanone heterocyclics such as ATHP, and 2-propoinyl-1-pyrroline (2PP) are unstable (liable) but important flavor compounds (Hofmann et al., 1998). These compounds are structural homologues and contain ring nitrogen and carbonyl oxygen atoms which can serve as possible binding sites to metal ions. However, to our knowledge only one example of complex of alkanone-heterocyclic can be found in the literature. 2-Acetylpyridine was shown to form a bivalent metal complex with zinc, copper, nickel or cobalt (Kidani et al., 1975). 2-Acetylpyridine has a conjugated ring system and it is very stable unlike the above mentioned compounds. The unstable nature of 2AP, ATHP and 2PP might limit the compounds’ abilities to form stable complexes with metal ions. However, there are no studies about the complexes of above mentioned compounds,
and the zinc-complexation has never been used intentionally for the stabilization of any flavoring compound.

2.9 References


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CHAPTER 3
CONVENIENT SYNTHESIS OF STABLE DEUTERIUM LABELED ALKYL PYRAZINES FOR USE IN STABLE ISOTOPE DILUTION ASSAYS

3.1 Abstract

Stable isotope dilution assays (SIDA) provide for accurate and precise quantitation of aroma components, such as alkylpyrazines, which are often present in low concentrations in complex food matrices. The unavailability of labeled standards is the main limitation to the widespread use in SIDA. This study describes the chlorination of several alkylpyrazines to form the corresponding chloro-alkylpyrazines which are efficient starting materials for the synthesis of deuterium labeled alkylpyrazines, namely [\(^2\)H\(_3\)]-2-methylpyrazine (d-1), [\(^2\)H\(_5\)]-2-ethylpyrazine (d-2), [\(^2\)H\(_5\)]-2,3(or 6)-dimethylpyrazine (d-3\(_A\), d-3\(_B\)), [\(^2\)H\(_3\)]-2,\([\(^2\)H\(_3\)]-6\)-dimethylpyrazine (d-3\(_C\)), [\(^2\)H\(_3\)]-2,\([\(^2\)H\(_3\)]-6\)-diethylpyrazine (d-4), [\(^2\)H\(_5\)]-2-ethyl-3(or 6)-methylpyrazine (d-5\(_A\), d-5\(_B\)), 2,\([\(^2\)H\(_3\)]-3,5\)-trimethylpyrazine (d-6), [\(^2\)H\(_5\)]-2-ethyl-3,6-dimethylpyrazine (d-7), [\(^2\)H\(_5\)]-2-ethyl-3,5-dimethylpyrazine(d-8), and 2,3-diethyl-[\(^2\)H\(_3\)]-5-methylpyrazine (d-9), which were obtained in good yields (57-100\%) and high purities (86-98\%). These stable isotopes were used as internal standards in SIDA to accurately and precisely determine selected alkylpyrazines in commercial peanut butter, cocoa powder, and instant coffee. 2,3-Diethyl-5-methylpyrazine (p-9) and 2-ethyl-3,5-dimethylpyrazine (p-8), despite their low abundance, had the highest odor-active values among the 13 pyrazines quantified in all products due to their very low odor thresholds.
3.2 Introduction

Pyrazines impart pleasant roasted and nut-like aroma notes to foods, and have been reported as predominant aroma components of cooked beef (Mussinan et al., 1973), French fries (Wagner et al., 1998), coffee (Czerny et al., 2000), and roasted peanuts (Burroni et al., 1997). Pyrazines have been used as food flavoring agents since the early 18th century, e.g., in preparation of an artificial coffee oil (Reichstein et al., 1928). Most naturally occurring pyrazines are generated during food preparation, so called reaction flavors, and often are used as indicators for monitoring the degree of processing/roasting for cocoa beans (Reineccius et al., 1976), coffee (Hashim et al., 1996), and peanut products (Buckholz Jr et al., 1980). Alkylpyrazine derivatives contain only hydrocarbon side groups. The annual use of naturally occurring alkylpyrazine derivatives in foods is estimated to be 300 tons and 860 kg for the artificial derivatives (Adams et al., 2002). Their odor thresholds for alkylpyrazines are in the range from 23 ppb for trimethylpyrazine (p-6) to 0.04 ppb for 2-ethyl-3,5-dimethylpyrazine (p-8) (Buttery et al., 1997).

Some sensorially relevant pyrazine compounds, such as 2,3-diethyl-5-methylpyrazine (p-9) and p-8, are often naturally present in low concentrations in foods. In general, foods represent very complex matrices containing high levels of proteins, sugars/carbohydrates, and fats. This makes quantitation of minor constituents, such as aroma components, rather difficult. Therefore, great care and special efforts are usually needed in order to obtain reliable quantitative results. Stable isotope dilution assay (SIDA) is a state-of-art quantitative method enabling both high precision and accuracy and has been successfully applied for the detection and quantitation of food contaminants (USFDA, 2013), food flavors
(Munch et al., 1998), and pesticide residues (Cai et al., 2004). The isotopically
labeled internal standard, which is spiked into the sample matrices prior to sample
preparation, has great similarity in both chemical and physical properties with its
unlabeled counterpart, which represents the target compound. After equilibration,
the ratio of natural substance (unlabeled target compound) and the labeled internal
standard is maintained throughout the entire analysis. The abundance of the target
analyte is determined by mass spectrometry in relation to the known amount of
isotope initially added to the sample.

The unavailability of labeled alkylpyrazines is the main limitation to the
widespread use of SIDA. Several attempts have been made to synthesize labeled
alkylpyrazines. Schieberle and Grosch condensed 2,3-pentanedione and \( [2H_4]\)-
ethylenediamine to obtain labeled 2-ethyl-3-methylpyrazine (\([2H_{1.2}]\)-p-5\(\Lambda\)) for use
in SIDA in wheat and rye bread crusts (Schieberle et al., 1987). Other deuterium
labeled alkylpyrazines, such as \([2H_3]\)-p-9, \([2H_3]\)-p-8, \([2H_3]\)-p-1, dimethylpyrazine
isomers (\([2H_3]\)-p-3\(A,B,C\)), and \([2H_3]\)-p-6, were prepared by nucleophilic addition via
the organolithium reagent, \([2H_3]\)-alkyl lithium, with the corresponding
alkylpyrazines. The target compounds were isolated in low yield via complicated
chromatography procedures such as HPLC or preparative GC (Cerny et al., 1993;
Chetschik et al., 2010). The main drawback of the above mentioned synthesis is the
generation of numerous side products, thus resulting in low overall yield of target
compounds. Organolithium reagent is highly reactive nucleophile, which
preferentially reacts with electrophilic substrates. However, due to the fact that an
alkylpyrazine is not an electrophilic substrate, this synthesis usually results in very
low yields.
Recently, our lab demonstrated the advantage of using chloropyrazines as substrates for the synthesis of three deuterated alkyl pyrazines (Fang et al., 2012). The present work describes the synthesis of various types of chloro-alkylpyrazines for use as starting materials for the preparation of twelve isotopically labeled alkylpyrazine compounds. The chlorine group on chloro-alkylpyrazines, acting as an electrophile, is attacked selectively and efficiently by a deuterated alkyl magnesium halide (Grignard reagent). This procedure promotes efficient production of isotopically labeled alkylpyrazines (Sato et al., 1996). The synthesized isotopes were used as internal standards in SIDA for the quantitation of selected alkylpyrazines in several types of foods. Two distinctly different extraction techniques for sample preparation were compared to access the precision and accuracy of SIDA.

3.3 Materials and methods

Peanut butter (Jif Creamy Peanut Butter 18 OZ; The J. M. Smucker Company, Orrville, OH), instant coffee (Maxwell House instant coffee original; Kraft Foods Global Inc., Northfield, IL), cocoa powder (Ghirardelli premium hot cocoa; Ghirardelli Chocolate Company, San Leandro, CA) were obtained from a local retailer (Champaign, IL). Phosphory chloride, [1,3-bis(diphenylphosphino) propane]nickel(II) chloride, hydrogen peroxide (35 wt. %), acetic acid, [2H3]-iodomethane (99.5+ atom % D), [2H5]-bromoethane (99 atom % D), vinylmagnesium bromide (1.0M in tetrahydrofuran), iodine, magnesium, 2-chloro-3,6-dimethylpyrazine (c-4), pyrazine, pentane, hexane, chloroform, deuterated chloroform (99.8 atom % D), methylene chloride, silica gel (grade 923, 100-200 mesh) were from Sigma-Aldrich.
(St Louis, MO). 2-Methylpyrazine (p-1), 2-ethylpyrazine (p-2), 2,3-dimethylpyrazine (p-3A), 2,6-dimethylpyrazine (p-3B), 2,5-dimethylpyrazine (p-3C), 2,3-diethylpyrazine (p-4A), 2-ethyl-3-methylpyrazine (p-5A), 2-ethyl-5(or 6)-methylpyrazine (p-5C, p-5B), 2,3,5-trimethylpyrazine (p-6), 2-ethyl-3,5(or 6)-dimethylpyrazine (p-8, p-7), and 2,3-diethyl-5-methylpyrazine (p-9), used in quantitative analysis as authentic standards (purity greater than 98%), were purchased from Sigma-Aldrich. Diethyl ether (anhydrous) was from Fisher Scientific (Fair Lawn, NJ). Chlorine (99.9%) was from Matheson (Basking Ridge, NJ).

3.3.1 Synthesis of 2-chloropyrazine

Pyrazine-1N-oxide (Klein et al., 1959): Pyrazine, acetic acid, and hydrogen peroxide in the mole ratio of 1 : 5 : 2 were placed into a reaction flask and heated at 75°C for 7 h. Reaction mixture was then alkalized (pH≥10, 20% NaOH) and extracted with methylene chloride. After removal of solvent, pyrazine-1N-oxide was obtained as fine white powder. GC retention indices (RI) (RTX-5) = 1750, RI (Innowax) = 2124; MS electron impact (EI), m/z (%) 96 (100, M+), 80 (67), 53 (50), 41 (33), 52 (32), 39 (31), 40 (27), 51 (21), 38 (13), 42 (6). UV absorption agreed with Klein et al (1959). 2-Chloropyrazine (c-1) (Klein et al., 1963): Pyrazine-1N-oxide was placed into a dry three-necked-flask equipped with a magnet stir bar and 2 mole ratio of phosphoryl chloride was added. The system was kept under nitrogen atmosphere to avoid moisture and maintained at 55°C for 45 min. When the reaction was complete, the reaction mixture was poured onto crushed ice and alkalized (20% NaOH). The solution was then extracted with diethyl ether (3 × 20 mL). The combined organic solution was passed through a short pad of silica gel.
and the solvent was dried over anhydrous sodium sulfate and removed through a Vigreux column (20 × 1 cm, 43°C) to obtain c-1 as clear colorless oil. GC RI (RTX-5) = 876, RI (Innowax) = 1432; MS(EI), m/z (%) 114 (100), 79 (81), 52 (46), 51 (34), 116 (33, M⁺), 60 (30), 87 (14), 62 (12), 53 (11), 38 (9).

### 3.3.2 Synthesis of 2,6-dichloropyrazine

Pyrazine-1N,4N-dioxide (Klein et al., 1959): Pyrazine, acetic acid, and hydrogen peroxide in the mole ratio of 1: 5 : 2 were heated at 95°C overnight. The acetic acid was evaporated by repeatedly heating and by addition of water. The residue was washed with hot chloroform (20 mL, ~60°C) to remove pyrazine-1N-oxide and then filtrated. The residue was washed with cold methanol (20 mL × 2), and then filtered. Pure pyrazine-1N,4N-dioxide was obtained as fine white powder.

2,6-Dichloropyrazine (c-2) (Klein et al., 1963): To pyrazine-1N,4N-dioxide, 3 mole ratio of phosphoryl chloride was added and heated at 70°C for 1 h. Then, reaction mixture was alkalized (20% NaOH) and extracted with diethyl ether. The organic solution was passed through a short pad of silica gel and the solvent was dried and removed to obtain c-2. GC RI (RTX-5) = 1373, RI (Innowax) = 1540; MS(EI), m/z (%) 148 (100, M⁺), 51 (83), 113 (82), 60 (76), 150 (63), 86 (56), 87 (34), 62 (30), 115 (24), 38 (20).

### 3.3.3 Synthesis of 2-chloro-3(or 6)-methylpyrazine

The synthesis method was modified from Gainer et al (1961). Chlorine gas was bubbled at a rate of 2 bubbles per second into 10 mL of carbon tetrachloride in test tube maintained in a -15 °C ice / salt bath for 60 min. P-1 (2 g) was then added.
The suspension was allowed to warm to room temperature for another 2 h. The white precipitate was collected and washed with carbon tetrachloride. After removing the solvent by heating at 50°C, 2.1 g of crude product was obtained. The white powder was then alkalized (20% NaOH), and then extracted with diethyl ether (15 mL × 3). The solvent layers were collected and the solvent removed using a Vigreux column (43°C). The residue was dissolved in mixed solvent of pentane and diethyl ether (90/10, P/E, v/v) passed through 10g of silica gel and eluted with same solvent (50 mL). Eluate was dried over anhydrous sodium sulfate and solvent was evaporated. A mixture of c-3A GC RI (RTX-5) = 967, RI (Innowax) = 1486; MS(EI), m/z (%) 93 (100), 128 (79, M+), 42 (53), 66 (39), 39 (30), 130 (26), 52 (24), 38 (22), 37 (17), 40 (16) and c-3B GC RI (RTX-5) = 972, RI (Innowax) = 1493; MS(EI), m/z (%) 128 (100, M+), 66 (59), 39 (46), 60 (39), 87 (38), 130 (33), 38 (25), 93 (25), 40 (24), 62 (16) was obtained. Purity = 98.2% (82.5% for c-3A, and 15.6% for c-3B, by GC-FID).

3.3.4 Synthesis of 2-chloro-3,5-dimethylpyrazine

2,6-Dimethylpyrazine-4N-oxide (Klein et al., 1959): A solution of p-3B, acetic acid, and hydrogen peroxide at a mole ratio of 1 : 9 : 1.5 was heated at 60°C in a round-bottom flask equipped with a magnet stir bar and condenser for 6 h. To minimize the formation of 2,6-dimethylpyrazine-1N,4N-dioxide, the temperature and heating time should not exceed 60°C and 6 h. When finished, this solution was cooled and alkalinized (20% NaOH) in an ice bath. The solution was then extracted with chloroform (15 mL × 3). Solvent layers were pooled and dried over anhydrous sodium sulfate. White needle-like crystals formed after evaporating the solvent
under reduced pressure at 50°C. The crystalline material was washed with boiling hexane then filtered (30 mL × 2) to obtain 2,6-dimethylpyrazine-4N-oxide, purity = 93.6% measured by GC-FID. GC RI (RTX-5) = 1277, RI (Innowax) = 2188; MS(EI), m/z (%) 42 (100), 124 (88, M⁺), 39 (68), 108 (66), 40 (50), 54 (35), 38 (26), 68 (18), 37 (13), 52 (13). 2-Chloro-3,5-dimethylpyrazine (c-5) (Klein et al., 1963): 2,6-Dimethylpyrazine-4N-oxide and phosphoryl chloride were place in a round-bottom flask at a mole ratio of 1: 3 at 60°C for 1 h. The reaction mixture was cooled, poured onto crushed ice, and pH adjusted (≧10, 20% NaOH). The dark solution was extracted with diethyl ether (20 mL × 3). Solvent layers were combined, dried and evaporated. The residue dissolved in mixed solvent (90/10, P/E, v/v), and then passed through a short pad of silica gel. The solvent was collected and evaporated, yielding c-5. Purity = 99% by (GC-FID). GC RI (RTX-5) = 1059, RI (Innowax) = 1556; MS(EI), m/z (%) 142 (100), 42 (90), 107 (79), 39 (72), 66 (72), 144 (32, M⁺), 38 (28), 40 (21), 37 (16 ), 143 (10).

3.3.5 Synthesis of 2,3-diethyl-5-chloropyrazine

A solution of p-4α (2g, 15 mmol), hydrogen peroxide (30 mmol), and acetic acid (0.15 mol) was heated at 65-75°C for 10.5 h. Then solution was cooled and alkalized (20% NaOH). After extraction with chloroform, solvent was dried and evaporated under reduced pressure. 1.8g of crude 2,3-diethylpyrazine-1N-oxide was obtained, yield 80%. GC RI (RTX-5) = 1748, RI (Innowax) = 2152; MS(EI), m/z (%) 135 (100), 119 (39), 152 (34, M⁺), 39 (26), 120 (24), 52 (20), 41 (17), 53 (17), 107 (16), 79 (15). C-6 was synthesized by adding crude 2,3-diethylpyrazine-1N-oxide (1.8g, 11 mmol) into phosphoryl chloride (88 mmol) in a round-bottom
flask at 65°C for 40 minutes. Then mixture was cooled and poured onto crushed ice. The solution was alkalinized and then extracted with diethyl ether (15 mL × 3). Solvent layers were collected and the solvent evaporated. The residue was separated via silica gel chromatography (10g, column: 15 cm length × 2.1 cm i.d.) conditioned with mixed solvent (90/10, P/E, v/v). After washing with same solvent, **C-6** was obtained in the first fraction (20 mL). GC RI (RTX-5) = 1223, RI (Innowax) = 1666; MS(EI), m/z (%) 170 (100), 155 (93), 80 (71), 169 (66), 39 (34), 172 (32, M⁺), 171 (31), 157 (27), 60 (26), 51 (25).

### 3.3.6 General procedure for the synthesis of alkylpyrazines

Grignard reagents: An etheric solution of deuterium labeled (or unlabeled) iodomethane (or bromoethane) was added by a syringe (dropwise) into a flask containing magnesium and a small amount of iodine in diethyl ether. The mixture was kept in an atmosphere of nitrogen for 2 h with good stirring at room temperature, and then cooled to 0-5°C. Alkylpyrazines: Freshly prepared Grignard reagent (120% mol) was placed under nitrogen purge, and [1,3-bis(diphenylphosphino)propane] nickel(II) chloride (Sato et al., 1996) as catalyst was added (1% mol). The chloro-alkylpyrazine in diethyl ether was then added dropwise by a syringe at 0°C during a period of 5 min. The reaction mixture was allowed to warm to room temperature for time period as described in **Table 3.1**. Then, water was added to quench the reaction. The solution was extracted with diethyl ether (3 × 15 mL). The combined solvent layers were washed with brine and then dried over anhydrous sodium sulfate and concentrated to 1 mL using a Vigreux column (43°C). The target compound was purified by silica gel
chromatography. Silica gel (10 g) was prepared in a column (15 cm length × 2.1 cm i.d.) and conditioned with a mixed solvent (90/10, P/E, v/v). Target compound was collected by eluting with a mixed solvent (90/10 ~ 70/30, P/E, v/v) and then was concentrated to 20 mL. After vacuum distillation/transfer (HVT), the distillate was dried over anhydrous sodium sulfate and solvent was removed by the means of Vigreux column (43°C) and nitrogen purge. An oil-like liquid with an appearance from colorless to yellow was obtained.

$[^3\text{H}_3]$-Methylpyrazine (d-1). GC RI (SAC-5) = 817, RI (Stabilwax) = 1252; MS(EI), m/z (%) 97 (100, M$^+$), 70 (60), 41 (29), 43 (27), 42 (25), 53 (21), 45 (19), 40 (15), 52 (13), 39 (12). $^1$H NMR: δ 8.47 (m, j = 1.7 Hz, 2H, 2-CH), 8.39 (d, j = 2.4 Hz, 1H, 1-CH). Isotope purity = 99.7%.

$[^3\text{H}_3]$-Ethylpyrazine (d-2). GC RI (SAC-5) = 906, RI (Stabilwax) = 1317; MS(EI), m/z (%) 111 (100), 113 (69, M$^+$), 81 (16), 112 (11), 41 (10), 61 (9), 53 (9), 52 (9), 84 (7), 42 (7). $^1$H NMR: δ 8.49 (m, j = 1.7 Hz, 2H, 2-CH), 8.40 (d, j = 2.5 Hz, 1H, 1-CH). Isotope purity = 99.5%.

$[^3\text{H}_2]$-[${^3\text{H}_3}$]-6-Dimethylpyrazine (d-3C). GC RI (RTX-5) = 876, RI (Innowax) = 1333; MS(EI), m/z (%) 46 (100), 114 (90, M$^+$), 41 (57), 43 (56), 42 (37), 40 (9), 39 (16), 38 (11), 70 (9), 44 (8). $^1$H NMR: δ 8.36 (s, 2H, 2-CH). Isotope purity = 99.2%.
Table 3.1 Synthesis, yields, purities, and reaction times of alkylpyrazine derivatives

<table>
<thead>
<tr>
<th>Grignard reagent from</th>
<th>Substrate ((D = {^2}\H))</th>
<th>product</th>
<th>Approximate(^a) reaction yield</th>
<th>Final(^b) yield</th>
<th>Purity %</th>
<th>Time(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD(_3)</td>
<td>(d-1)</td>
<td>~84%</td>
<td>14%</td>
<td>86.2</td>
<td>1 d</td>
</tr>
<tr>
<td></td>
<td>C(_2)D(_5)</td>
<td>(d-2)</td>
<td>~84%</td>
<td>12%</td>
<td>96.3</td>
<td>6 h</td>
</tr>
<tr>
<td>Cl-N-Cl</td>
<td>CD(_3)</td>
<td>(d-3(_c))</td>
<td>~75%</td>
<td>42%</td>
<td>91.4</td>
<td>3 d</td>
</tr>
<tr>
<td>Cl-N-Cl</td>
<td>C(_2)D(_5)</td>
<td>(d-4)</td>
<td>~100%</td>
<td>35%</td>
<td>93.4</td>
<td>3 h</td>
</tr>
<tr>
<td>(c-3(_a)) + (c-3(_b))</td>
<td>CD(_3)</td>
<td>(d-3(_a))</td>
<td>~92%</td>
<td>22%</td>
<td>97.1(^c)</td>
<td>3 d</td>
</tr>
<tr>
<td></td>
<td>C(_2)D(_5)</td>
<td>(d-5(_a))</td>
<td>~100%</td>
<td>55%</td>
<td>98.2(^c)</td>
<td>3 h</td>
</tr>
<tr>
<td>Cl-N-Cl</td>
<td>CD(_3)</td>
<td>(d-6)</td>
<td>~75%</td>
<td>25%</td>
<td>96.6</td>
<td>3 d</td>
</tr>
<tr>
<td>Cl-N-Cl</td>
<td>C(_2)D(_5)</td>
<td>(d-7)</td>
<td>~100%</td>
<td>82%</td>
<td>98.0</td>
<td>3 h</td>
</tr>
<tr>
<td>Cl-N-Cl</td>
<td>C(_2)D(_5)</td>
<td>(d-8)</td>
<td>~100%</td>
<td>23%</td>
<td>91.6</td>
<td>3 h</td>
</tr>
<tr>
<td>Cl-N-Cl</td>
<td>CD(_3)</td>
<td>(d-9)</td>
<td>~88%</td>
<td>40%</td>
<td>91.2</td>
<td>29 h</td>
</tr>
</tbody>
</table>

\(^a\) Approximate reaction yield is based on the area ratio of [product(s) / (starting material(s) + product(s))] \times 100% on GC-MS; \(^b\) Final yield: The mole yield after purification; \(^c\) Purity is the amount of total isomers; \(^d\) Times are not optimized.
[\textsuperscript{2}H\textsubscript{5}]\textsuperscript{-}2,[\textsuperscript{2}H\textsubscript{5}]\textsuperscript{-}6-Diethylpyrazine (d-4). GC RI (SAC-5) = 1067, RI (Stabilwax) = 1411; MS(EI), m/z (%) 144 (100), 146 (56, M\textsuperscript{+}), 114 (18), 145 (13), 41 (12), 57 (8), 62 (7), 42 (7), 147 (5), 113 (4). \textsuperscript{1}H NMR: δ 8.34 (s, 2H, 2-CH). Isotope purity = 99.2%.

[\textsuperscript{2}H\textsubscript{5}]\textsuperscript{-}2,3-Dimethylpyrazine (d-3\textsubscript{A}). GC RI (SAC-5) = 911, RI (Stabilwax) = 1331; MS(EI), m/z (%) 111 (100, M\textsuperscript{+}), 67 (40), 70 (37), 42 (15), 40 (14), 112 (12), 43 (11), 41 (10), 45 (10), 52 (5). \textsuperscript{1}H NMR: δ 8.29 (s, 2H, 2-CH), 2.55 (s, 3H, 1-CH\textsubscript{3}). Isotope purity = 98.9%.

[\textsuperscript{2}H\textsubscript{5}]\textsuperscript{-}2,6-Dimethylpyrazine (d-3\textsubscript{B}). GC RI (SAC-5) = 906, RI (Stabilwax) = 1313; MS(EI), m/z (%) 111 (100, M\textsuperscript{+}), 43 (46), 45 (33), 40 (20), 39 (16), 41 (13), 42 (10), 112 (7), 38 (5), 84 (4). \textsuperscript{1}H NMR: δ 8.27 (s, 2H, 2-CH), 2.53 (s, 3H, 1-CH\textsubscript{3}). Isotope purity = 99.5%.

[\textsuperscript{2}H\textsubscript{5}]\textsuperscript{-}2-Ethyl-3-methylpyrazine (d-5\textsubscript{A}). GC RI (SAC-5) = 990, RI (Innowax) = 1386; MS(EI), m/z (%) 127 (100, M\textsuperscript{+}), 125 (69), 126 (55), 67 (24), 42 (19), 84 (16), 40 (12), 41 (11), 86 (11), 95 (11). \textsuperscript{1}H NMR: δ 8.34 (d, j = 2.5 Hz, 1H, 1-CH), 8.29 (d, j = 2.7 Hz, 1H, 1-CH), 2.58 (s, 3H, 1-CH\textsubscript{3}). Isotope purity = 97.0%.

[\textsuperscript{2}H\textsubscript{5}]\textsuperscript{-}2-Ethyl-6-methylpyrazine (d-5\textsubscript{B}). GC RI (SAC-5) = 990, RI (Innowax) = 1365; MS(EI), m/z (%) 125 (100), 127 (64, M\textsuperscript{+}), 95 (17), 39 (14), 126 (12), 61 (11), 41 (10), 40 (9), 42 (8), 43 (7). \textsuperscript{1}H NMR: δ 8.34 (d, j = 2.5 Hz, 1H, 1-CH), 8.29 (d, j = 2.7 Hz, 1H, 1-CH), 2.55 (s, 3H, 1-CH\textsubscript{3}). Isotope purity = 97.0%. (A mixed
solution of $\text{d-5}_A$ (88%) and $\text{d-5}_B$ (10%) isomers. The $^1$H NMR signal of $\text{d-5}_B$ may be covered due to its low abundance relative to $\text{d-5}_A$.

2,[$^2$H$_3$]-3,5-Trimethylpyrazine (d-6). GC RI (SAC-5) = 995, RI (Stabilwax) = 1387; MS(EI), m/z (%) 125 (100, M$^+$), 42 (94), 45 (42), 39 (31), 40 (22), 81 (21), 57 (11), 38 (10), 126 (9), 43 (9). $^1$H NMR: δ 8.16 (s, 1H, 1-CH), 2.50 (s, 3H, 1-CH$_3$), 2.49 (s, 3H, 1-CH$_3$). Isotope purity = 97.4%.

[$^2$H$_5$]-2-Ethyl-3,6-dimethylpyrazine (d-7). GC RI (SAC-5) = 1072, RI (Stabilwax) = 1427; MS(EI), m/z (%) 141 (100, M$^+$), 139 (76), 140 (57), 42 (41), 61 (19), 39 (19), 110 (13), 109 (13), 40 (11), 108 (10). $^1$H NMR: δ 8.18 (s, 1H, 1-CH), 2.56 (s, 3H, 1-CH$_3$), 2.52 (s, 3H, 1-CH$_3$). Isotope purity = 98.3%.

[$^2$H$_5$]-2-Ethyl-3,5-dimethylpyrazine (d-8). GC RI (SAC-5) = 1077, RI (Stabilwax) = 1442; MS(EI), m/z (%) 141 (100, M$^+$), 139 (71), 140 (56), 61 (37), 42 (26), 39 (19), 110 (11), 40 (11), 109 (10), 142 (9). $^1$H NMR: δ 8.20 (s, 1H, 1-CH), 2.54 (s, 3H, 1-CH$_3$), 2.49 (s, 3H, 1-CH$_3$). Isotope purity = 99.2%.

2,3-Diethyl-5-[${^2}$H$_3$]-methylpyrazine (d-9). GC RI (SAC-5) = 1150, RI (Stabilwax) = 1476; MS(EI), m/z (%) 153 (100, M$^+$), 138 (74), 152 (62), 57 (31), 124 (25), 41 (17), 154 (10), 39 (8), 43 (7), 139 (7). $^1$H NMR: δ 8.20 (s, 1H, 1-CH), 2.82 (dq, j = 1, 7.5 Hz, 4H, 2-CH$_2$), 1.28 (dt, j = 1, 7.5 Hz, 6H, 2-CH$_3$). Isotope purity = 99.5%.
2,6-Diethylpyrazine (p-48). Synthesized from ethyl-magnesium-bromide and c-2. Purity (94.8%) was checked by GC-FID. GC RI (SAC-5) =1072, RI (Stabilwax) = 1420; MS(EI), m/z (%) 135 (100), 136 (51, M+), 39 (13), 108 (11), 53 (10), 56 (7), 107 (6), 54 (5), 120 (5), 52 (5). 1H NMR: δ 8.32 (s, 2H, 2-CH), 2.86 (q, j = 7.6 Hz, 4H, 2-CH2), 1.34 (t, j = 8.0 Hz, 6H, 2-CH3).

2-Vinyl-3,6-dimethylpyrazine (p-10). Synthesized from c-4 (0.5 g, 3.5 mmol), triethylamine (0.5g, 5 mmol), and vinylmagnesium bromide (5 mmol). Large amount of triethylamine was necessary to prevent pyrazine dimer (or polymer) formation. Purity = 94.4% (GC/FID). GC RI (SAC-5) = 1169, RI (Stabilwax) = 1525; MS(EI), m/z (%) 133 (100), 134 (65, M+), 42 (45), 39 (27), 54 (20), 40 (12), 66 (8), 65 (7), 135 (5), 52 (5). 1H NMR: δ 8.21 (s, 1H, 1-CH), 6.96 (q, jav = 9 Hz, 1H, 1-CH), 6.42 (cis) (dd, j = 17, 1 Hz, 2H, 1-CH2), 5.58 (trans) (dd, j = 11, 1 Hz, 2H, 1-CH2), 2.57 (s, 3H, 1-CH3), 2.52 (s, 3H, 1-CH3).

2-Ethyl-3,6-dimethylpyrazine (p-7). Synthesized from c-4 and ethyl-magnesium-bromide (120% mol). Note: commercially available ethyl-dimethylpyrazine is a mixture of p-7 and p-8. Purity = 99.4% (GC/FID). GC RI (RTX-5) = 1083, RI (Innowax) = 1469; MS(EI), m/z (%) 135 (100), 136 (71, M+), 42 (58), 39 (44), 56 (23), 108 (18), 40 (18), 41 (17), 53 (11). 1H NMR: δ 8.17 (s, 1H, 1-CH), 2.82 (q, j = 7.6 Hz, 2H, 1-CH2), 2.54 (s, 3H, 1-CH3), 2.51 (s, 3H, 1-CH3), 1.28 (t, j = 7.6 Hz, 3H, 1-CH3).
3.3.7 Compound identification

Deuterium labeled compounds were identified by comparing their mass spectra with unlabeled authentic standard compounds. For example, Fig. 3.1 and Fig. 3.2 show a similar pattern of for the EI mass spectra of the two isotopologues. Note that molecular weight is shifted by several units due to the higher mass of deuterium. $^1$H NMR was also applied for further confirmation. The missing signals from deuterated side chains on labeled pyrazine compounds indicate the positions of deuterium compared to the authentic unlabeled standard compounds (as shown in Fig. 3.3).

3.3.8 Compound purities

The purities of the deuterated compounds were determined by gas chromatography coupled with a flame-ionization detector (GC-FID) using a 5890 Series II GC (Agilent Technologies Inc., Palo Alto, CA). Compounds were injected directly into GC inlet (250°C, hot split, 50:1). Purities were obtained by calculating target peak area divided by total area. Separations were performed using an Innowax column (30 m × 0.25 mm ×0.25 μm, Agilent Technologies Inc.). Oven was programmed from 40°C to 225°C at the rate of 10°C /min and holding at the end for 10 minutes. Isotope purities for deuterated compounds were based on the integrated area of selected side chain groups as follows: isotope purity (%) = [(1H signal for authentic standard - 1H residue for the deuterated compound) / (1H signal for authentic standard)] x 100%.
Figure 3.1 MS(EI) of unlabeled (a) and of \([\text{H}_3]\)-labeled 2-ethyl-3,5-dimethylpyraizne (b).
Figure 3.2 MS(EI) of unlabeled (a) and of $[\text{H}_3]$-labeled 2,3-diethyl-5-methylpyrazine (b).
Figure 3.3 $^1$H NMR spectra of (a) and of $[^2]$H$_5$-labeled 2-ethyl-3,6-dimethylpyrazine (b).
3.3.9 $^1$H Nuclear magnetic resonance (NMR)

Compounds were prepared in deuterated chloroform (C$_2$HCl$_3$) in WILMAD 528-PP 5 mm NMR tubes. NMR spectra were acquired on a Varian Unity 500 spectrometer (499.693 MHz), equipped with a 5mm Nalorac QUAD probe (1H, 19F, 13C, 31P). The probe temperature was 20.4°C for the proton spectra. Chemical shift $\delta$ are cited in parts per million (ppm) in reference to CHCl$_3$ as 7.26 ppm. The multiplicity abbreviations used to describe NMR signals are $s =$ singlet, $d =$ doublet, $t =$ triplet, $q =$ quartet, $m =$ multiplet, $dd =$ double doublet, $dt =$ double triplet, $dq =$ double quartet, $j =$ j coupling, and $jav =$ average of j coupling.

3.3.10 Quantitation of selected pyrazines

Compound identification in the quantitative study was based on matching retention indices on both polar (Stabilwax or Innowax) and non-polar (RTX-5 or SAC-5) columns and on comparison of mass spectra to authentic standards. The concentrations of selected pyrazines in food samples were based on their area response ratio corrected by response factors (Table 3.2), as follows: mass of analyte = [(extracted ion chromatogram area of analyte) / (area of labeled internal standard) $\times$ mass of internal standard $\times$ response factor]. Response factors were determined by analyzing mixtures of known amounts of the unlabeled and labeled compounds at five different mass ratios (1:10, 1:5, 1:1, 5:1, and 10:1) by GC-MS.
Table 3.2 Pyrazines, the respective isotopologues, selected ions and (EI)-MS response factors used in stable isotope dilution assays

<table>
<thead>
<tr>
<th>Pyrazine</th>
<th>Labeled standard</th>
<th>Mass trace (m/z)</th>
<th>Response factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>analyte</td>
<td>Internal standard</td>
</tr>
<tr>
<td>2-methyl-</td>
<td>(p-1)</td>
<td>[1H3]-methyl-</td>
<td>(d-1)</td>
</tr>
<tr>
<td>2-ethyl-</td>
<td>(p-2)</td>
<td>[1H3]-ethyl-</td>
<td>(d-2)</td>
</tr>
<tr>
<td>2,3-dimethyl-</td>
<td>(p-3A)</td>
<td>[1H3]-2,3-dimethyl-</td>
<td>(d-3A)</td>
</tr>
<tr>
<td>2,6-dimethyl-</td>
<td>(p-3B)</td>
<td>[1H3]-2,6-dimethyl-</td>
<td>(d-3B)</td>
</tr>
<tr>
<td>2,5-dimethyl-</td>
<td>(p-3C)</td>
<td>[1H3]-2,5-dimethyl-</td>
<td>(d-3C)</td>
</tr>
<tr>
<td>2,3-diethyl-</td>
<td>(p-4A)</td>
<td>[1H10]-2,3-diethyl-</td>
<td>(d-4)</td>
</tr>
<tr>
<td>2,6-diethyl-</td>
<td>(p-4B)</td>
<td>[1H10]-2,6-diethyl-</td>
<td>(d-4)</td>
</tr>
<tr>
<td>2-ethyl-3-methyl-</td>
<td>(p-5A)</td>
<td>[1H3]-2-ethyl-3-methyl-</td>
<td>(d-5A)</td>
</tr>
<tr>
<td>2-ethyl-6-methyl-</td>
<td>(p-5B)</td>
<td>[1H3]-2-ethyl-6-methyl-</td>
<td>(d-5B)</td>
</tr>
<tr>
<td>2-ethyl-5-methyl-</td>
<td>(p-5C)</td>
<td>[1H5]-2-ethyl-5-methyl-</td>
<td>(d-5C)</td>
</tr>
<tr>
<td>2,3,5-trimethyl-</td>
<td>(p-6)</td>
<td>2,[1H3]-3,5-trimethyl-</td>
<td>(d-6)</td>
</tr>
<tr>
<td>2-ethyl-3,6-dimethyl-</td>
<td>(p-7)</td>
<td>[1H3]-2-ethyl-3,6-dimethyl-</td>
<td>(d-7)</td>
</tr>
<tr>
<td>2-ethyl-3,5-dimethyl-</td>
<td>(p-8)</td>
<td>[1H5]-2-ethyl-3,5-dimethyl-</td>
<td>(d-8)</td>
</tr>
<tr>
<td>2,3-diethyl-5-methyl-</td>
<td>(p-9)</td>
<td>[1H3]-2,3-diethyl-5-methyl-</td>
<td>(d-9)</td>
</tr>
<tr>
<td>2-vinyl-3,6-dimethyl-</td>
<td>(p-10)</td>
<td>[1H5]-2-ethyl-3,6-dimethyl-</td>
<td>(d-7)</td>
</tr>
</tbody>
</table>
3.3.11 Direct solvent extraction - solvent assisted flavor evaporation (DSE-SAFE)

A sample (100 g) of peanut butter, cocoa powder, or instant coffee, was stirred in 100 mL saturated aqueous sodium chloride solution, and then 150 mL diethyl ether was added for the purpose of extraction. Isotopically labeled internal standards in diethyl ether were added (based on preliminary experiments). This mixture was stirred for 3 h to achieve equilibrium between the internal standards and analytes. After centrifuging, the solvent layer was collected and the residue was extracted two more times with 100 mL of diethyl ether (1 h solvent contact time for each extraction). The combined solvent extracts were concentrated to 150 mL using a Vigreux column (43°C) prior to SAFE. Volatile compounds were collected after distillation at $5 \times 10^{-5} \sim 9 \times 10^{-5}$ Torr for 3 h as previously described (Engel et al., 1999). The SAFE apparatus was kept at 45°C with a circulating water bath. The SAFE distillate was concentrated to 30 mL, and then fractionated to neutral/basic (NB) fraction and acidic (AC) fractions (Potsatchakul et al., 2008). Due to the fact that pyrazines are weakly basic compounds, only the NB fraction was analyzed by GC-MS. Sample extraction/analysis was performed in duplicate.

3.3.12 Solid phase micro-extraction (SPME)

A sample, consisting of 0.3 g to 10 g of peanut butter, cocoa powder, or instant coffee was placed in a 22-mL vial containing 0.6 g of NaCl and 3 mL of water. The vial was spiked with a 20 μL mixture of internal standards and sealed with aluminum crimp cap and Teflon-lined silicon septum. SPME was performed using a divinylbenzene / carboxen / polidymetylosiloxane (DVB / CAR / PDMS) fiber
(Supleco, Bellefonte, PA). The sample vial was incubated at 60°C for 20 min for equilibration and then the SPME fiber was exposed to the headspace of the sample for 30 min. Volatiles were transferred immediately via the injection port (hot splitless; 260 °C; 4 min valve-delay) of the gas chromatograph and desorbed for 10 min for subsequent analysis by GC-MS.

### 3.3.13 Gas chromatography-mass spectrometry (GC-MS)

GC-MS was performed using a 6890 GC-5973N mass selective detector (Agilent Technologies Inc.). Separations were performed on a Stabilwax column (30 m x 0.25 mm x 0.25 μm film thickness, Restek, Bellefonte, PA). Helium was the carrier at a constant flow of 1 mL/min. To minimize loss of any labile constituents, DSE-SAFE extracts were injected using a CIS4 (Gerstel GmbH & Co. KG, Germany) programmable temperature vaporization (PTV) inlet in the cold splitless mode (-50°C for 0.1 min, then ramped at 12°C/sec and held at 260°C). GC oven temperature was programmed from 35 to 225°C at 6°C/min with initial and final hold times of 5 and 20 min, respectively. Other conditions were as follows: MSD interface temperature, 260°C; ionization energy, 70 eV; mass range, 35-350 a.m.u; EM voltage, Autotune + 165 V; scan rate, 4.45 scans/s.

### 3.4 Results and discussion

Reaction of chloro-alkylpyrazines with deuterium labeled alkyl Grignard reagents provides an improved method for the preparation of isotopically labeled alkylpyrazines. The chloro-alkylpyrazine compounds used as starting materials
were synthesized by either direct chlorination or by chlorination of alkylpyrazine-N-oxide compounds.

3.4.1 Direct Chlorination

Passing chlorine gas through a carbon tetrachloride (CCl₄) solution of p-1 resulted in a dense white precipitate being formed. The precipitate was the hydrochloride salt of c-3ᴬ and c-3ᴮ. This chlorination was not a free radical process, but an electrophilic substitution (Hirschberg et al., 1961). During chlorination, the inductive effect of the positively charged nitrogen atom in the pyrazine-perchloride complex caused the nearby carbons on p-1 to be relatively electronegative, thus the chlorination occurred on the 3 carbon or 6 carbon. The same approach could be applied in case of the chlorination of the 2,5-disubstituted pyrazine compounds (Hirschberg et al., 1961). However, treating the 2,6-disubstituted pyrazines or the 2,3-disubstituted pyrazines directly with chlorine in CCl₄ solution appeared to be a free radical process and, thus, the chlorination occurred on the alkyl side chain (Hirschberg et al., 1961). Since alkylpyrazines containing side chain halogens cannot be used as starting materials for the purpose of preparation of labeled pyrazines, another approach was developed for the chlorination of the 2,6-disubstituted pyrazines.

3.4.2 Chlorination of 2,6-dimethylpyrazine

As mentioned above, the direct chlorination of p-3ᴮ formed a side chain halogenated product. To obtain a ring chlorinated product, p-3ᴮ was treated with hydrogen peroxide in glacial acetic acid. During the oxidation process, a mixture of
**p-3_b.** 2,6-dimethylpyrazine-1N-oxide, and 2,6-dimethylpyrazine-4N-oxide was formed at the ratio of 19 : 47 : 34 (by GC-FID). This mixture was purified by washing with boiling hexane. Because 2,6-dimethylpyrazine-4N-oxide has a high melting point (m.p. ~ 110°C) (Klein et al., 1959) and low solubility in hexane, it can be collected after filtering. On the other hand, **p-3_b** and 2,6-dimethylpyrazine-1N-oxide (m.p. ~ 55°C) (Klein et al., 1959) passed through the filter. The reaction of 2,6-dimethylpyrazine-4N-oxide with phosphory chloride first resulted in the formation of a N-O-dichlorophosphite. Then, the electron-deficient carbon (adjacent to the nitrogen) was attacked by chloride, followed by oxygen removal (Klein et al., 1963) to form **c-5** (Scheme 3.1).

(Scheme 3.1) Synthesis of [\(^{2}\text{H}_5\)]-2-ethyl-3,5-dimethylpyrazine.
(Scheme 3.2) Synthesis of 2,3-diethyl-[^3]H_3]-5-methylpyrazine.

3.4.3 Chlorination of 2,3-diethylpyrazine

Ohta et al. (1982) reported the N-oxidation and chlorination of some 2,3-disubstituted pyrazines such as dimethylpyrazine. Two relevant products, 2,3-dimethyl-5-chloropyrazine and 2-α-chloromethyl-3-methylpyrazine, were identified in that study. In the present work we started from p-4\_A. The oxidation process gave a single product: 2,3-diethylpyrazine-1N-oxide. After chlorinating, two main products with m/z 144 were found which were separated and purified into two fractions assumed to be c-6 and 2-α-chloroethyl-3-ethylpyrazine (scheme 3.2). To confirm this, the first fraction was treated with methyl magnesium iodine. A single product of 2,3-diethyl-5-methylpyrazine (p-9) was found for which the RI and mass spectrum matched the authentic standard compound. This confirmed our earlier assumptions. Treating c-6 with [^3]H_3]-methyl-magnesium-iodine in diethyl ether for 29 h afforded deuterium labeled d-9 (yield = 88%). D-9 is an important flavor contributor in many foods due to its very low odor threshold (OT) of 0.5 ppb (Wagner et al., 1998).
3.4.4 Deuterated alkylpyrazines

Twelve deuterated alkylpyrazines were synthesized using this convenient synthesis approach. The reactions are shown in Table 3.1. The average reaction yield was approximately 87%. The high yield was due to efficient and selective reaction of the Grignard reagent with the chloro-alkylpyrazines. The unreacted starting material (chloro-alkylpyrazine) was easy to separate from the desired labeled product (deuterated alkylpyrazine) because the chlorine group made these two compounds very different with respect to their elution through silica gel chromatography column. Chloropyrazine was not held by silica gel in a mixed solvent (90/10, P/E, v/v). Meanwhile, the labeled alkylpyrazines required a more polar mobile phase (80/20 ~ 70/30, P/E, v/v) to be eluted from the column. Therefore, the labeled alklypyrazines could be obtained in high purities.
Table 3.3 Concentrations and OAVs for selected alkylpyrazines in food products by two extraction techniques: SAFE (solvent-assisted flavor evaporation) and SPME (solid-phase microextraction).

<table>
<thead>
<tr>
<th>alkylpyrazines</th>
<th>Odor threshold (µg kg⁻¹)</th>
<th>Peanut butter</th>
<th>Instant coffee</th>
<th>Cocoa powder</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Oil</td>
<td>SAFE</td>
<td>SPME</td>
</tr>
<tr>
<td>p-8</td>
<td></td>
<td></td>
<td>80±3.3⁴</td>
<td>66±2.6</td>
</tr>
<tr>
<td>p-9</td>
<td>0.04ᵃ</td>
<td>2.2ᵇ</td>
<td>12±0.2</td>
<td>11±1.0</td>
</tr>
<tr>
<td>p-7</td>
<td>0.5ᵇ</td>
<td>0.5ᵇ</td>
<td>328±2.0</td>
<td>348±30</td>
</tr>
<tr>
<td>p-6</td>
<td>23ᵃ</td>
<td>297ᵈ</td>
<td>475±3.6</td>
<td>442±0.5</td>
</tr>
<tr>
<td>p-3ᶜ</td>
<td>1700ᵃ</td>
<td>2600ᵈ</td>
<td>1426±45</td>
<td>1524±72</td>
</tr>
<tr>
<td>p-5ᶜ</td>
<td>100ᵃ</td>
<td>900ᶜ</td>
<td>336±7.0</td>
<td>329±5.0</td>
</tr>
<tr>
<td>p-5ᵇ</td>
<td>NRᵉ</td>
<td>900ᶜ</td>
<td>83±3.4</td>
<td>81±0.0</td>
</tr>
<tr>
<td>p-3ᵇ</td>
<td>1500ᵃ</td>
<td>8000ᵉ</td>
<td>272±26</td>
<td>258±18</td>
</tr>
<tr>
<td>p-2</td>
<td>4000ᵃ</td>
<td>1700⁰</td>
<td>142±7.7</td>
<td>140±11</td>
</tr>
<tr>
<td>p-3ᵃ</td>
<td>2500ᵃ</td>
<td>NR⁰</td>
<td>88±2.5</td>
<td>87±0.4</td>
</tr>
<tr>
<td>p-1</td>
<td>2700⁰</td>
<td>2700ᵉ</td>
<td>462±2.6</td>
<td>459±21</td>
</tr>
<tr>
<td>p-10</td>
<td>NR</td>
<td>NR</td>
<td>103±6.3</td>
<td>116±9.1</td>
</tr>
<tr>
<td>p-4ᵇ</td>
<td>6ᵃ</td>
<td>NR</td>
<td>8±0.3</td>
<td>7±0.4</td>
</tr>
</tbody>
</table>

a) Buttery et al., 1997; b) Wagnar et al., 1998; c) Koehler et al., 1971; d) Chetschik et al., 2010; e) Fors, 1983;
f) NR, not reported; g) ND, not detected; h) OAV, odor active values calculated based on SAFE extraction; i) mean±SD, n=2. (µg kg⁻¹)
3.4.5 Quantitative analysis

Peanut butter, instant coffee, and cocoa powder were chosen for quantitative studies based on their complex matrices and because pyrazines are known to be important aroma components of these products (Matsui et al., 1998; Blank et al., 1992; Frauendorfer et al., 2002). Samples were prepared by solid phase micro-extraction (SPME) and solvent assisted flavor evaporation (SAFE) in order to evaluate the breadth and utility of the SIDA technique.

Concentrations and odor-active values (OAVs) for selected alkylpyrazines in commercial peanut butter, instant coffee, and cocoa powder are given in Table 3.3. The quantitative data in the literature concerning alkylpyrazines in peanut products was limited, and most of these reports focused only on the highly abundant alkylpyrazines, such as \( p-3_c \) and \( p-3_b \) (Leunissen et a., 1996; Baker et al., 2003). However, these highly abundant alkylpyrazines are not considered to be major contributors to roasted peanut flavor because of their relatively OTs (Schirack et al., 2006). On the other hand, certain pyrazines with low OTs, such as \( p-7 \), \( p-8 \), and \( p-9 \) are reported to be important odorants despite their relatively low abundance in peanut products (Schirack et al., 2006). Previously, \( p-7 \), \( p-8 \), and \( p-9 \) were determined to be 352 ng/g, 5534 ng/g, and 2.2 ng/g (Baker et al., 2003), respectively, in blanched oven roasted peanuts (by use of an unlabeled internal standard) and 196 ng/g, 23 ng/g, and 13 ng/g, respectively, in pan roasted peanuts (by SIDA) (Chetschik et al., 2010). The above concentrations are consistent with those determined in the present study for the commercial peanut butter, although some differences due to peanut variety, processing and other factors are to be expected. In addition, low standard deviations (<10%) were observed in the SIDA
of pyrazines in pan roasted peanuts (Chetschik et al., 2010) and in the present study. Compounds \( \text{P-8} \) and \( \text{p-9} \) were reported to have the highest flavor dilution (FD) factors in coffee (Blank et al., 1992), which agrees with the results of this study in which \( \text{p-8} \) and \( \text{p-9} \) had the highest OAVs among all pyrazines measured in instant coffee. Pyrazines comprised over 40% of the cocoa powder essence. Among them, dimethylpyrazine isomers (DMPs: \( \text{p-3}_A, \text{p-3}_B, \text{p-3}_C \)) and trimethylpyrazine (TrMP, \( \text{p-6} \)) were present in highest abundance. The DMPs/TrMP index is often used to assess the degree of cocoa roasting (Bonvehi et al., 2005). \( \text{P-3}_A, \text{p-3}_B, \text{p-3}_C, \) and \( \text{p-6} \) were found to be the most abundant alkylpyrazines in cocoa powder in the present study. On the other hand, \( \text{p-8}, \text{p-9}, \text{p-7}, \) and \( \text{p-6} \) were reported to have high FD factors of 2048, 256, 256, and 128, respectively, over other compounds by molecular sensory analysis of cocoa powder (Frauendorfer et al., 2006). This agrees with the results of the present study, where the OAVs of these compounds were determined to be 1500, 113, 18, and 13, respectively. Therefore, SIDA appears to be a suitable means to accurately quantify alkylpyrazines in food products.

A crucial step in the determination of compounds which are responsible for the flavor of a food product is to select a suitable extraction method that allows for the extraction of all compounds and does not alter the flavor profile of characteristic volatiles. To test the breadth of SIDA, two different extraction/isolation methods solvent-assisted flavor evaporation (SAFE) and solid-phase micro-extraction (SPME) were tested in this study. The average difference between SAFE and SPME was less than 10% (4.9%). Only three single pyrazines (\( \text{p-3}_C \) in instant coffee, \( \text{p-6} \) and \( \text{p-8} \) in peanut butter) differed significantly (\( p<0.05, \) t-test)(Table 3.4). The precision of SIDA was also acceptable, with an average relative standard
deviation (%RSD) of 3.6%, which ranged from 0.0% to 12.6%. The higher RSD for p-9 was probably due to its low concentration (~10 µg kg\(^{-1}\)). Some other errors could have been caused by the difficulty in precisely spiking trace amounts of the labeled internal standards or by inconsistent integration of very small peaks during data analysis.

Table 3.4 Differences and P-values of pyrazines quantified in food products by SAFE and SPME

<table>
<thead>
<tr>
<th>alkylpyrazines</th>
<th>Peanut butter</th>
<th>Instant coffee</th>
<th>Cocoa powder</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Difference(^a)</td>
<td>P (t-test)(^b)</td>
<td>Difference(^a)</td>
</tr>
<tr>
<td>p-8</td>
<td>18.44</td>
<td>0.044</td>
<td>11.06</td>
</tr>
<tr>
<td>p-9</td>
<td>2.87</td>
<td>0.794</td>
<td>1.58</td>
</tr>
<tr>
<td>p-7</td>
<td>5.82</td>
<td>0.460</td>
<td>1.65</td>
</tr>
<tr>
<td>p-6</td>
<td>7.15</td>
<td><strong>0.006</strong></td>
<td>2.35</td>
</tr>
<tr>
<td>p-3(_C)</td>
<td>6.66</td>
<td>0.112</td>
<td>6.90</td>
</tr>
<tr>
<td>p-5(_C)</td>
<td>2.09</td>
<td>0.181</td>
<td>2.60</td>
</tr>
<tr>
<td>p-5(_B)</td>
<td>2.97</td>
<td>0.930</td>
<td>0.11</td>
</tr>
<tr>
<td>p-3(_B)</td>
<td>5.21</td>
<td>0.634</td>
<td>0.04</td>
</tr>
<tr>
<td>p-2</td>
<td>1.97</td>
<td>0.494</td>
<td>5.10</td>
</tr>
<tr>
<td>p-3(_A)</td>
<td>1.58</td>
<td>0.098</td>
<td>10.43</td>
</tr>
<tr>
<td>p-1</td>
<td>0.56</td>
<td>0.998</td>
<td>3.23</td>
</tr>
<tr>
<td>p-10</td>
<td>11.26</td>
<td>0.607</td>
<td>-</td>
</tr>
<tr>
<td>p-4(_B)</td>
<td>9.43</td>
<td>0.249</td>
<td>2.86</td>
</tr>
</tbody>
</table>

\(a\) Difference = [Absolute value of (value of SAFE – value of SPME) / AVERAGE of values of SAFE and SPME] \times 100%

\(b\) P = Possibility (n = 2)

3.5 References

Adams, T. B.; Doull, J.; Feron, V. J.; Goodman, J. I.; Marnett, L. J.; Munro, I. C.; Newberne, P. M.; Portoghese, P. S.; Smith, R. L.; Waddell, W. J.; Wagner, B.


Chetschik, I.; Granvogl, M.; Schieberle, P. Quantitation of key peanut aroma compounds in raw peanuts and pan-roasted peanut meal. Aroma reconstitution


CHAPTER 4

STABILIZATION OF THE POTENT ODORANT 2-ACETYL-1-PYRROLINE AND ITS HOMOLOGUES BY COMPLEXATION WITH ZINC HALIDES

4.1 Abstract

2-Acetyl-1-pyrroline (2AP) and the structurally similar compounds 6-acetyl-2,3,4,5-tetrahydropyrdine (ATHP, along with its tautomer 6-acetyl-1,2,3,4-tetrahydropyridine), 2-propionyl-1-pyrroline (2PP), and 2-acetyl-2-thiazoline (2A2T) are well-known potent odorants in various food products. However, due to the highly unstable nature of these compounds, especially 2AP and ATHP, they are scarcely used commercially in flavor formulations. A novel and attractive method for stabilization of these potent odorants in dry powder form is presented. Coordination of 2AP, ATHP, 2PP and 2A2T to zinc ions (ZnI₂, ZnBr₂ or ZnCl₂) resulted in the formation in high yields of stable crystalline complexes, which upon hydration release the free odorant. Infrared spectroscopy was used to study the coordination complexes. 2AP contains donor atoms which coordinate (with covalent character) through both the heterocyclic nitrogen and carbonyl oxygen atoms to the zinc ion. This is also the case for ATHP, 2PP and 2AT, but not for 2A2T, since the sulfur group in 2A2T provides a third possible donor site. Stability studies showed that the 2AP-zinc iodide complex (with 14% loading) maintained greater than 94% retention of 2AP after 3 months of storage at ambient temperature.
in a dry environment. Meanwhile, the ATHP-ZnI₂ complex was similarly stable and retained 88% of the ATHP after 2 months of storage. This stabilization technology may enable the commercial use of this powerful aroma compound as a flavoring agent.

4.2 Introduction

2-Acetyl-1-pyrroline (2AP) was first identified in cooked rice (Buttery et al., 1982) and was regarded as the most powerful odorant among all rice volatiles identified (Buttery et. al., 1983). 2AP, with an odor threshold of 0.1µg L⁻¹ in water (Buttery et al., 1982), dominates its homologues 6-acetyl-2,3,4,5-tetrahydropyrdine (ATHP, including tautomer 6-acetyl-1,2,3,4-tetrahydropyridine), 2-propionyl-1-pyrroline (2PP), and 2-acetyl-2-thiazoline (2AT), all of which possess roasted, cracker-like, and popcorn-like aroma notes and are also important odorants in numerous food products (Adams et al., 2006).

The specialty fragrant or aromatic rice varieties contain high levels of 2AP. In an effort to boost the production of 2AP, the gene linked to the accumulation of 2AP in fragrant rice have been identified (Bradbury et al., 2005) and later transferred into non-aromatic rice varieties, which enabled them to accumulate greater levels of 2AP (Vanavichit et al., 2008). 2AP is of potential commercial interest to the flavor industry because of its pleasant and characteristic flavor. However, the highly unstable nature of the compound has hindered its widespread commercial use. In popcorn 2AP decreased by about 80% during room temperature storage for one week in polyethylene bags (Schieberle, 1995).
aromatic rice, the concentration of 2AP was shown to diminish by about half its original content after 3 months (Widjaja et al., 1996). The instability of 2AP has been postulated to occur via a polymerization reaction (Buttery et al., 1982). The cyclic imine of 1-pyrroline was shown to form trimers during work-up which then undergo condensation with neighboring molecules to form polymeric products (Fuhlhage et al., 1958).

Some efforts have been made to increase the stability of 2AP. Duby et al. (1996) patented a process for encapsulation of 2AP with β-cyclodextrin (β-CD); however, 2AP (10% loading) was decomposed by 91% after 13 days of storage at 20°C. At a loading of 1%, 2AP decomposed by 99% after 110 days of storage. Additional efforts to stabilize 2AP were made by Srinivas et al. (2006) through encapsulation, where gum acacia and/or starch were applied to form a stabilized dry powder; however, no proven stability was demonstrated. In another study, a crude extract from pandan leaves served as a natural source 2AP which was mixed with β-CD to form a powder. However, no storage stability data was reported to demonstrate the effectiveness of this approach (Andreas et al., 2012). In a different study 2AP was maintained at a level of 70% after 72 days of storage at ambient temperature when encapsulated at very low loading (0.003%) in a gum acacia/maltodextrin matrix (Apintanapong et al., 2003).

Previous attempts to stabilize 2AP suffer to some extent from at least one of the following three deficiencies: 1) stability of 2AP was either not mentioned or was insufficiently studied; 2) low loadings of 2AP were used; and 3) 2AP was only stable during storage at low temperature (4°C and -20°C). In this study, a novel and attractive stabilization method is described. The compound, 2AP is coordinated as a
ligand onto a zinc halide, thus forming a powdered stable complex. Complexes of 2AP, as well as some other heterocycles such as 2PP, ATHP, 2A2T, 2-acetylpyrazine (APra), 2-acetylpyridine (APri), and 2-acetylthiazole (ATz), can be prepared with zinc chloride, zinc bromide and zinc iodide in a similar manner. The chemical characteristics of these complexes and stability of the 2AP-zinc iodide complex during storage described.

4.3 Materials and methods

4.3.1 Chemicals

2-Acetyl-2-thiazoline (2A2T), 2-acetylthiazole (2ATz), 2-acetylpyridine (2APri), 2-acetylpyrazine (2APry), piperidine, pyrrolidine, collidine, nonane, zinc chloride (1.0 M solution in diethyl ether), zinc bromide, iodomethane, bromoethane, magnesium (turnings, \(\sim\)1/8 in.), zinc (dust, <10\(\mu\)m), iodine, boric acid and zinc monosodium salt were purchased from Sigma-Aldrich (St Louis, MO, USA). 2A2T was purified by precipitating out impurities in anhydrous diethyl ether, and by subsequent distillation \textit{in vacuo}. Zinc iodide was prepared by stirring zinc and iodine in anhydrous diethyl ether for 3 days. Anhydrous diethyl ether and methylene chloride were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Starch was from EM Science (Gibbstown, NJ, USA) and was baked overnight at 200°C prior to use. 2AP (yield = 10% from pyrrolidine; purity = 91%) and ATHP (yield = 5% from piperidine; purity = 83% with two tautomers 6-acetyl-2,3,4,5-tetrahydropyridine and 6-acetyl-1,2,3,4-tetrahydropyridine at a ratio of 7 : 3) were synthesized using published methods (De Kimpe et al., 1993a; 1993b). 2-Propionyl-1-pyrroline was prepared by reacting 2-cyano-1-pyrroline with
ethylmagnesium bromide in diethyl ether, by following closely the published procedure for the synthesis of 2-acetyl-1-pyrroline (yield = 4% from pyrrolidine; purity = 42%). 2-Ethyl-1-pyrroline was synthesis as described by Fuganti et al. (2007).

4.3.2 General procedure for the preparation of alkanone-heterocyclic-zinc halide complexes

An anhydrous etheric solution of 2AP or other alkanone-heterocyclic compounds (1 mmol in 10 mL ether) was added to zinc iodide, zinc bromide or zinc chloride in anhydrous diethyl ether (0.1M) in a 50-mL centrifuge tube with good stirring and nitrogen purging. During addition, a significant amount of precipitate was observed. The reaction mixture was stored at -20°C for 5 min, and then centrifuged (3000 × g, 3 min). The precipitate was washed with fresh anhydrous diethyl ether (10 mL × 3), and then collected. Solvent was removed by gentle purging with nitrogen to obtain a fine powdered complex which was stored at -20 °C in a vial equipped with a PTFE-lined silicon cap.

4.3.3 Quantitation of alkanone-heterocyclic compound content of complexes

Complex (5-10 mg) was placed in a 1.5 mL vial, then 0.5 mL of aqueous phosphate buffer (50 mM, pH = 7) was added (this solution turned cloudy/white because of the insoluble metal content). Subsequent extraction was conducted via addition of methylene chloride (0.5 mL) containing an internal standard (collidine, 1.00 mg mL⁻¹). This mixture was vigorously shaken by hand for 1 min, and then centrifuged (3000 × g, 5 min). The solvent layer was recovered and dried over
sodium sulfate prior to GC analysis. Authentic standard alkanone-heterocyclic compounds were dissolved in phosphate buffer and extracted with methylene chloride following the above procedure to generate response factors for each alkanone-heterocyclic compound against the internal standard. The content of an alkanone-heterocyclic compound in a complex was calculated using the following equation:

\[
\text{Loading (\%) = area of alkanone-heterocyclic compound peak / area of collidine peak} \times \text{concentration of collidine (mg mL}^{-1}) \times 0.5 \text{ mL} \times \text{response factor / sample weight (mg)} \times 100\%.
\]

### 4.3.4 Stability of 2AP with zinc iodide in solution

2AP (2000μg mL\(^{-1}\)) in pentane (1 mL) and zinc iodide diethyl ether solution (0.2M, 0.2 mL) were combined with 8 mL of methylene chloride (containing nonane as internal standard at 1.00 mg mL\(^{-1}\)) in a 10-mL volumetric flask and the volume immediately made up to the mark with methylene chloride. The solution was incubated at room temperature for a period of 20 h during which time the 2AP content was periodically monitored by transferring a 0.5 mL aliquot of the sample solution into a 1.5 mL vial followed by addition of a 0.5 mL quenching solution (phosphate buffer, 50 mM, pH = 7). The mixture was shaken and centrifuged (3000 ×g, 5 min) and the solvent layer was immediately analyzed by GC. To check the stability of the quenched 2AP extract, the initial sample (time zero, quenched immediately after zinc iodide addition) was held at room temperature and analyzed periodically by GC for up to 20 h. To serve as a control, a separate 2-AP solution
which did not contain zinc iodide was also stored at room temperature and monitored periodically during this same time period.

4.3.5 Gas chromatography (GC)

Gas chromatography was performed using either a 1) 6890N GC/5973N mass selective detector (MSD; Agilent Technologies Inc., Palo Alto, CA) equipped with a fused-silica capillary column (Stabilwax, 30 m × 0.25 mm × 0.25 μm film thickness, or Rtx-5, 15 m × 0.32 mm × 0.5 μm film thickness, Restek, Bellefonte, PA); or 2) 5890 series II GC equipped with a split/splitless injector, flame ionization detector (FID) and HP-1 column (30m× 0.32 mm × 0.25 μm film thickness, Agilent). Helium was the carrier at a constant flow of 1 mL/min. The injector was held at 250 °C in the split mode. GC oven temperature was programmed from 50 to 225°C at 10°C/min with initial and final hold times of 5 and 20 min, respectively. Other conditions for the MSD were as follows: MSD interface temperature, 260°C; ionization energy, 70 eV; mass range, 35-350 a.m.u; EM voltage, Autotune + 165 V; scan rate, 4.45 scans/s.

4.3.6 Determination of zinc content in alkanone-heterocyclic-zinc complexes

Zinc content was measured spectrophotometrically using a published procedure (Säbel et al., 2010). Samples (5 to 10 mg) were diluted in water (pH = 2, adjusted using HCl) in a 25-mL volumetric flask. A 70 μL aliquot of this solution, plus 100 μL of Zincon dye (1.6 mM; 37 mg Zincon in 1 mL NaOH (1M) prior to dilute into 50 mL water) and 3 mL borate buffer (53 mM, pH=9.0), were combined in a test tube. After incubation of this solution for 5 min, absorbance was measured at 620
nm using a Lambda 1050 UV/VIS/NIR spectrophotometer (PerkinElmer Inc., Shelton, CT). A six-point calibration plot was constructed from a dilution series of a zinc stock solution (100 mg zinc dust in 1 mL HCl for 4 h prior to dilution to 1 L).

4.3.7 Infrared spectra

Infrared spectra of the alkanone-heterocyclic compounds and their zinc halide complexes were recorded using Spectrum 100 FT-IR (PerkinElmer Inc.) with Attenuated Total Reflectance (ATR) accessory (Resolution = 4 cm⁻¹, 4 scans/spectra, range = 500cm⁻¹ ~ 4000cm⁻¹). Liquid Samples were analyzed in neat form. Solid complexes were applied directly to the diamond ATR top-plate.

4.3.8 Stability of 2AP zinc iodide complex in dry powder form

2AP zinc iodide complex (5 to 10 mg) was stored in sealed (PTFE-lined silicon cap) 1.5-mL glass vials and stored at 25°C, 10°C or -20°C. For low moisture storage, complex was either placed in a desiccator under vacuum and dessicant (calcium chloride) or mixed with five times (by weight) starch (final = 1.9%) which served as an anticaking/desiccating agent. This mixture was stored normally (not in desiccator) at 25°C. The residual 2AP in complex was monitored during a 3 month storage period as previously described.

4.4 Results and discussion

4.4.1 Complexation of alkanone-heterocyclic compounds to zinc halides

The addition of anhydrous zinc iodide to a dry solution of 2AP in diethyl ether caused a yellowish precipitate to form immediately. This reaction mixture was
incubated in the freezer for 5 min, and then triturated with fresh anhydrous diethyl ether to remove excess zinc iodide and other impurities from the 2AP-zinc iodide complex. A fine powder, pale-yellow in color, was obtained after collecting the precipitate and purging with nitrogen. The free 2AP ligand could be freed from the powdered 2AP-zinc iodide complex via hydration with water in which case a white cloudy metallic substance also appeared at neutral pH and the yellow color faded immediately. GC-MS analysis of a methylene chloride extract of this aqueous mixture yielded a single peak with a mass spectrum and retention index closely matching those of authentic 2AP.

The 2AP-zinc iodide complex acts to stabilize 2AP through the coordination of the ring nitrogen and carbonyl oxygen atoms of the compound to the zinc ion. Coordination of 2AP with other zinc salts such as zinc bromide and zinc chloride acts in a similar fashion; however, during the preparation of the 2AP-zinc chloride complex, a white sticky precipitate formed initially and later a hard layer formed. This reaction mixture was centrifuged and then triturated with fresh anhydrous diethyl ether during which the sticky precipitate was converted into a powder. In the case of zinc chloride complex the trituration step is essential, since upon standing it will transform into a red viscous material which might be due to the polymerization of 2AP. This same phenomenon occurred in the preparation of the 2-PP-zinc iodide, -zinc bromide and -zinc chloride complexes and for the 2-ethyl-1-pyrroline-zinc iodide complex, but was not observed for the other alkanone-heterocyclic-zinc halide complexes.
4.4.2 Stoichiometry of complex formation

Contents of alkanone-heterocyclic compounds and zinc metal in the various complexes were determined by GC and spectrophotometric analyses, respectively. The theoretical loadings of 2AP were 26%, 33%, and 45% in the zinc iodide, zinc bromide and zinc chloride complexes, respectively. However, we found the content of 2AP was, respectively, only 12-17%, 11%, 13% in the zinc iodine, zinc bromide, and zinc chloride complexes. The lower loadings could be due to the instability of 2AP which may have degraded during the complexation step. Similar phenomena were observed for the complexes of 2PP and ATHP, which are both unstable cyclic imines. Higher recoveries could be obtained by optimizing the method of preparation. Table 4.1 shows the yields, color, proposed stoichiometry, and content analysis of 21 alkanon-heterocyclic-zinc halide complexes. 2A2T, APri, APra, and ATz had values close to their calculated alkanone-heterocyclic compound and metal contents. For 2AP, 2PP, and ATHP, only the zinc contents were used for the determination of their proposed stoichiometry, due to the poor recovery of the alkanone-heterocyclic compounds.

Zinc halide was postulated to be have two binding sites in its complex (Baxter et al., 1991); however, due to its flexible coordination, four or even six chelate rings were also reported (Erxleben, 2003). Because 2AP, 2PP and ATHP contained two donors atoms (a nitrogen and a carbonyl oxygen), they were postulated to form 1-to-1 metal-ligand complexes. However, although APri contained two donors as well, it was found to form bivalent metal complexes (Kidani, et al., 1975) and, accordingly, the APri-zinc complexes were found to have 4 binding sites. 2A2T and APra were believed to form (ZnX$_2$)$_3$L$_2$ complexes (where, X=I, Br or Cl;
L=APra ligands) since both had three possible donor sites (nitrogen, carbonyl, and sulfur atoms (Weaver, et al., 1970) for 2-AT; two nitrogens and a carbonyl for APra). However the same rule did not apply to ATz, for which 1 to 1 complexes were found even though ATz contains three donor atoms. The same ligands or structurally similar ligands that link to zinc halide may have various tetrahedral center, and (pyrazine)$_2$-ZnBr$_2$ which forms 2D regular square-grid coordinations. For example, pyrazine zinc bromide could yield two reversible coordination polymers, pyrazine-ZnBr$_2$ which forms zigzag chain polymer with a network with octahedral center (Bourne et al., 2001). Further work such as X-ray diffraction may useful in elucidating the complex multidimensional structures of alkanone-heterocyclic complexes or even correct the proposed stoichiometry. However, this is beyond the scope of the present study.
Table 4.1 Yields, colors, stoichiometry, and content analyses of alkanone-heterocyclic-zinc halide complexes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Yield(^a)</th>
<th>Color</th>
<th>(^b)Stoichiometry (n)</th>
<th>Found (calcd.) (%)</th>
<th>Heterocyclic</th>
<th>Zinc halide</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnI(_2)(2-Acetyl-1-pyrrole)(_n)</td>
<td>62%</td>
<td>pale yellow</td>
<td>1</td>
<td>17±2 (26)</td>
<td>73±2 (74)</td>
<td></td>
</tr>
<tr>
<td>ZnBr(_2)(2-Acetyl-1-pyrrole)(_n)</td>
<td>96%</td>
<td>white</td>
<td>1</td>
<td>11±1 (33)</td>
<td>67±2 (67)</td>
<td></td>
</tr>
<tr>
<td>ZnCl(_2)(2-Acetyl-1-pyrrole)(_n)</td>
<td>86%</td>
<td>white</td>
<td>1</td>
<td>13±1 (45)</td>
<td>57±13 (55)</td>
<td></td>
</tr>
<tr>
<td>ZnI(_2)(2-Propionyl-1-pyrrole)(_n)</td>
<td>32%</td>
<td>orange</td>
<td>1</td>
<td>8±1 (28)</td>
<td>76±2 (72)</td>
<td></td>
</tr>
<tr>
<td>ZnBr(_2)(2-Propionyl-1-pyrrole)(_n)</td>
<td>49%</td>
<td>pale yellow</td>
<td>1</td>
<td>13±1 (36)</td>
<td>65±5 (64)</td>
<td></td>
</tr>
<tr>
<td>ZnCl(_2)(2-Propionyl-1-pyrrole)(_n)</td>
<td>60%</td>
<td>pale yellow</td>
<td>1</td>
<td>23±2 (48)</td>
<td>53±3 (52)</td>
<td></td>
</tr>
<tr>
<td>ZnI(_2)(2-Acetyl-2-thiazoline)(_n)</td>
<td>90%</td>
<td>yellow</td>
<td>2/3</td>
<td>25±2 (21)</td>
<td>77±5 (79)</td>
<td></td>
</tr>
<tr>
<td>ZnBr(_2)(2-Acetyl-2-thiazoline)(_n)</td>
<td>95%</td>
<td>white</td>
<td>2/3</td>
<td>33±1 (28)</td>
<td>72±2 (72)</td>
<td></td>
</tr>
<tr>
<td>ZnCl(_2)(2-Acetyl-2-thiazoline)(_n)</td>
<td>98%</td>
<td>Pale yellow</td>
<td>2/3</td>
<td>47±11 (39)</td>
<td>61±2 (61)</td>
<td></td>
</tr>
<tr>
<td>ZnI(_2)(6-Acetyl-2,3,4,5-tetrahydropyridine)(_n)</td>
<td>59%</td>
<td>yellow</td>
<td>1</td>
<td>7±1 (28)</td>
<td>71±11 (72)</td>
<td></td>
</tr>
<tr>
<td>ZnBr(_2)(6-Acetyl-2,3,4,5-tetrahydropyridine)(_n)</td>
<td>71%</td>
<td>pale yellow</td>
<td>1</td>
<td>10±0 (36)</td>
<td>68±1 (64)</td>
<td></td>
</tr>
<tr>
<td>ZnCl(_2)(6-Acetyl-2,3,4,5-tetrahydropyridine)(_n)</td>
<td>100%</td>
<td>pale yellow</td>
<td>1</td>
<td>25±2 (48)</td>
<td>56±6 (52)</td>
<td></td>
</tr>
<tr>
<td>ZnI(_2)(2-Acetylpyridine)(_n)</td>
<td>102%</td>
<td>white</td>
<td>2</td>
<td>42±2 (43)</td>
<td>59±1 (57)</td>
<td></td>
</tr>
<tr>
<td>ZnBr(_2)(2-Acetylpyridine)(_n)</td>
<td>97%</td>
<td>white</td>
<td>2</td>
<td>52±2 (52)</td>
<td>51±7 (48)</td>
<td></td>
</tr>
<tr>
<td>ZnCl(_2)(2-Acetylpyridine)(_n)</td>
<td>101%</td>
<td>white</td>
<td>2</td>
<td>58±2 (64)</td>
<td>36±0 (36)</td>
<td></td>
</tr>
<tr>
<td>ZnI(_2)(2-Acetylpyrazine)(_n)</td>
<td>74%</td>
<td>orange</td>
<td>2/3</td>
<td>25±0 (20)</td>
<td>79±8 (80)</td>
<td></td>
</tr>
<tr>
<td>ZnBr(_2)(2-Acetylpyrazine)(_n)</td>
<td>93%</td>
<td>white</td>
<td>2/3</td>
<td>30±2 (27)</td>
<td>73±1 (73)</td>
<td></td>
</tr>
<tr>
<td>ZnCl(_2)(2-Acetylpyrazine)(_n)</td>
<td>91%</td>
<td>white</td>
<td>2/3</td>
<td>37±1 (37)</td>
<td>65±5 (63)</td>
<td></td>
</tr>
<tr>
<td>ZnI(_2)(2-Acetylthiazole)(_n)</td>
<td>40%</td>
<td>pale yellow</td>
<td>1</td>
<td>27±0 (29)</td>
<td>72±1 (72)</td>
<td></td>
</tr>
<tr>
<td>ZnBr(_2)(2-Acetylthiazole)(_n)</td>
<td>76%</td>
<td>white</td>
<td>1</td>
<td>36±0 (36)</td>
<td>64±1 (64)</td>
<td></td>
</tr>
<tr>
<td>ZnCl(_2)(2-Acetylthiazole)(_n)</td>
<td>103%</td>
<td>white</td>
<td>1</td>
<td>47±2 (48)</td>
<td>54±1 (52)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Yield = \(W / (mM \times MW / n) \times 100\%\) \(W = \) amount (mg) of obtained complex; \(mM = \) amount (mM) of starting alkanone-heterocyclic; \(MW = \) molecular weight of complex (base on the formula in this table); \(^b\) Proposed stoichiometry; \(^c\) 6-Acetyl-2,3,4,5-tetrahydropyridine comes with its tautomer 6-acetyl-1,2,3,4-tetrahydropyridine.
Table 4.2 Infrared spectral assignment (cm$^{-1}$) of alkanone-heterocyclics and their zinc complexes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Carbonyl Vibration (cm$^{-1}$)</th>
<th>Peak shape</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free ligand</td>
<td>ZnI$_2$ complex</td>
</tr>
<tr>
<td>2-acetyl-1-pyrroline</td>
<td>1695</td>
<td>1627</td>
</tr>
<tr>
<td>2-propoinyl-1-pyrroline</td>
<td>1698</td>
<td>1647</td>
</tr>
<tr>
<td>6-acetyl-2,3,4,5-tetrahydropyridine</td>
<td>1695, 1666</td>
<td>1632</td>
</tr>
<tr>
<td>2-acetyl-2-thiazoline</td>
<td>1700</td>
<td>1694</td>
</tr>
<tr>
<td>2-acetyl-1-thiazole</td>
<td>1682</td>
<td>1656</td>
</tr>
<tr>
<td>2-acetyl-1-pyrazine</td>
<td>1688</td>
<td>1674</td>
</tr>
<tr>
<td>2-acetyl-1-pyridine</td>
<td>1696</td>
<td>5659</td>
</tr>
</tbody>
</table>

4.4.3 Infrared spectra and nature of coordination

The infrared spectra of the Zn(II)-heterocyclic complexes were recorded in the range 4000-400 cm$^{-1}$, but only 2000-600 cm$^{-1}$ region was considered. The carbonyl (C=O) band assignments for the complexes are given in Table 4.2. It was observed that the carbonyl vibration shifted to lower wavenumbers for the complexes as compared to their free ligands, indicating the existence of a coordination complex. This was observed for all complexes except for those of 2A2T. For example, 2AP produced a very strong absorption around 1695 cm$^{-1}$ which was attributed to C=O stretching. The corresponding bends shifted to the lower wavenumbers at 1627 cm$^{-1}$,
1630 cm\(^{-1}\), and 1633 cm\(^{-1}\), for its zinc iodide, zinc bromide, and zinc chloride complexes, respectively. This provides evidence for coordination through the carbonyl oxygen. In addition, the broadened band width at around 1695 cm\(^{-1}\) in the complexes may be considered as further evidence for metal chelation (Singh et al., 2002). The heterocyclic ring nitrogen was suggested to undergo bond formation with the metal, thus increasing the dipolar contribution of C=N\(^+\) (Kidani et al., 1975). Bands at 1632 cm\(^{-1}\) or 1619 cm\(^{-1}\) may be assigned as ring nitrogen vibration. However, the C-C vibration occurred at around 1600 to 1650 cm\(^{-1}\) with broad and split or multi-peak shapes which always overlapped the nitrogen (C-N=C) region, which made it more difficult to determine the coordination between the metal and ring nitrogen. We believed nitrogen was involved in coordination because many heterocyclic-metal complexes contained only nitrogen and carbon have been previously observed, such as (1-pyrroline)\(_2\)-zinc iodide (Baxter et al., 1991), (pyridine)\(_2\)-zinc chloride, (pyridine)\(_6\)-cadmium chloride (Mason, et al., 1925), pyrazine-zinc bromide (Bourne et al., 2001), and (2-ethyl-1-pyrroline)\(_2\)-zinc iodide which was observed in our laboratory.

For ATHP, the carbonyl vibration located at 1695 cm\(^{-1}\) and 1666 cm\(^{-1}\) in the free ligand, disappeared upon O-complexation, and shifted to lower wavenumbers of 1632 cm\(^{-1}\), 1635 cm\(^{-1}\), and 1637 cm\(^{-1}\) in its zinc iodide, zinc bromide, and zinc chloride complexes, respectively. The two bands for ATHP indicated the carbonyl groups of two tautomers. However, there was only one band observed in their complexes which suggested a single stable ATHP-zinc halide complex was constructed. However, for 2A2T complexes, the differences between coordinated and non-coordinated carbonyl groups was evidenced by only the broadening and
splitting of this band which was located at around 1700 cm\(^{-1}\). A strong C=N stretching band at 1592 cm\(^{-1}\) (Doornbos et al., 1972) shifted to lower wavenumbers at 1574 cm\(^{-1}\), 1576 cm\(^{-1}\), 1576 cm\(^{-1}\) for its zinc iodide, zinc bromide, and zinc chloride complexes, respectively, apparently indicating N coordination.

**4.4.4 Unstable nature of 2AP**

Lower loadings were observed in the complexes of 2AP as well in 2PP and ATHP. This could due to the instability of imines which decayed to some extent during complexation. It was observed that 2AP zinc iodide complex solutions prepared in methanol, acetone, dimethyl sulfoxide (DMSO), propylene glycol, or glycerin turned red intermediately, but solutions of the free ligand of 2AP in these solvents were colorless or pale-yellow. It was furthermore considered that zinc may catalyze the polymerization reaction because 2AP in these solvents was relatively stable but instable in the presence of zinc ion. The 2AP-zinc iodide complex in tetrahydrofuran (THF), chloroform, and methylene chloride (polarity = 3 ~ 4) was yellow in color and turned red gradually. A sticky dark-red material which adhered to the wall of the beaker was observed due to the poor solubility of the polymer. In non-polar solvents such as pentane, benzene, and diethyl ether, the complex remained a solid powder. The stabilization seemed to happen in relatively non-polar solvents, such as diethyl ether, because precipitation was necessary in order to prevent zinc-mediated polymerization. No precipitate was formed when 2AP (200 μg mL\(^{-1}\)) treated with zinc iodide (4 mM) in methylene chloride for 20 h. A quenching solution (phosphate buffer, 50 mM, pH=7, 0.5 mL) was added to 0.5 mL of sample solution prior to GC analysis which not only quenching zinc-mediated
polymerization but also minimizing the chromatographic interference by zinc. The results showed 2AP decayed 70% in 4 h (Fig. 4.1) when zinc ion was present. However, when a 2AP/zinc ion solution was treated with the quench solution within 1 minute, 2AP was retained at 97% and maintained stable during a 20 h period. The results indicated zinc ion catalyzed the deterioration of 2AP.

Figure 4.1 2-Acetyl-1-pyrroline (2AP) degradation in methylene chloride solution presented zinc iodine. (mean±SD, n=2)
4.4.5 Stability of 2-AP zinc iodide complex

The tendency of metal-ligands coordination complexes to form can be applied for the protection of unstable compounds, such as the stabilization of 1-pyrroline as a stable crystalline complex with zinc ion (Baxter et al., 1991)(Freer et al., 1993). The stability of 2AP zinc iodide complex was studied over a period of three months. A complex containing 14.5% 2AP showed a 74% deduction in 2AP after 78 days of storage at 25°C (Fig 4.2). As a comparison, a 0.17% aqueous solution of 2AP (pH=7) showed 80% reduplication after 3 days and 90% after 5 days. These results suggested that the complexation process increased the stability of 2-AP and may be applied to extend the shelf-life of commercial use of 2-AP as flavoring agent.

Figure 4.2 2-Acetyl-1-pyrroline (2AP) reduction of its zinc iodide complex during storage at various temperatures. (mean±SD, n=2)
As expected, lower temperature storage was found to favor the complex stability. During storage at -20°C, the 2AP content was maintained at 97% in the complex (content = 12.5%) after 92 days of storage. However, surprisingly, 96% retention was observed after 78 days of storage when the 2AP complex (14.5%) was stored at 10°C. It was considered that there might be another factor in addition to temperature that influenced the stability of the complex during storage. The 2AP complex which was stored at 25°C was observed to be caked, while samples stored at 10°C remained a free-flowing powder. It was considered that trace amounts of moisture which penetrated the vials during storage might accelerated 2AP degradation. Once 2AP lost the protection of the complexation, it would decay via self-polymerization. In this case, refrigerate may work like a dehumidifier, thus providing a low moisture environment. To prove this, samples of 2AP complex were stored in a desiccator or mixed with starch. The results (Fig. 4.3) show that the stability was significantly increased when moisture was excluded during storage. The 2AP content in complex remained at 67% after 92 days storage when the complex was distributed in starch. Meanwhile, in a moisture free environment, 2AP (14.4% complex) was observed to decline by only 6% after 3 months of storage at 25°C. The ATHP-ZnI₂ complex was similarly stable and only 12% of the ATHP was lost after 2 months of storage at 25°C.
Figure 4.3 2-Acetyl-1-pyrroline (2AP) reduction of its zinc iodide complex stored in low moisture conditions at 25°C. (mean±SD, n=2)

4.5 Conclusions

Complexation of unstable 2AP with zinc iodide forms a solid complex in powder form. This complex is stable at ambient temperature (only 6% reduction during a 3 months of storage at 25°C) which may enable its use as a flavoring agent. When applying this complex to foods, the temperature, moisture, and other possible food matrix components may influence its stability. Therefore, an appropriate package or encapsulation technology, such as dispersion in an edible oil or lipid or wax encapsulation is suggested in order to protect the complex from moisture and
interactions with other food components. However, use of zinc chloride may be preferred since it has GRAS status. This novel stabilization process, hopefully, can enable the practical use of the labile, yet powerful flavor compounds 2-acetyl-1-pyrroline and its homologues as flavoring agents.

4.6 References


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224.
Accurate determination of compounds which are responsible for the flavor of a food product is critical in order to understand the aroma profile of that food. Stable isotope dilution assays (SIDA) provide for accurate and precise quantitation of aroma components, but unavailability of labeled standards has been a main limitation to the widespread use of the method. This study developed a convenient synthesis method for the preparation of stable isotopically labeled alkylpyrazines to be used as internal standards for SIDA.

In the present study, in order to optimize the synthesis yield of deuterium labeled alkylpyrazines, several alkylpyrazines were chlorinated beforehand to form the chloroalkylpyrazines which are efficient starting materials for the synthesis. Twelve isotopes, namely $[^2\text{H}_3]-2$-methylpyrazine, $[^2\text{H}_3]-2$-ethylpyrazine, $[^2\text{H}_3]-2,3$-(or 6)-dimethylpyrazine, $[^2\text{H}_3]-2,[^3\text{H}_3]-6$-dimethylpyrazine, $[^3\text{H}_3]-2,[^3\text{H}_3]-6$-diethylpyrazine, $[^2\text{H}_3]-2$-ethyl-3-(or 6)-methylpyrazine, $[^2\text{H}_3]-2,[^3\text{H}_3]-3,5$-trimethylpyrazine, $[^2\text{H}_3]-2$-ethyl-3,6-dimethylpyrazine, $[^2\text{H}_3]-2$-ethyl-3,5-dimethylpyrazine, and 2,3-diethyl-$[^2\text{H}_3]-5$-methylpyrazine, which were obtained in good yields (57-100%) and high purities (86-98%). These stable isotopes were used for SIDA to determine selected alkylpyrazines in commercial peanut butter, cocoa powder, and instant coffee. Two different extraction/isolation methods, solvent-assisted flavor evaporation (SAFE), and solid-phase micro-extraction (SPME) were applied to test the breadth of this method. The average difference and relative standard deviation
(%RSD) between SAFE and SPME was less than 10% (4.9%) and 3.6%, respectively. It was found that two pyrazines, 2,3-diethyl-5-methylpyrazine and 2-ethyl-3,5-dimethylpyrazine, though present at the lowest concentrations, showed the highest odor-activities in all products tested due to their very low odor detection thresholds.

Stability is critical in order for a flavor compound to be applied in a food product. 2-Acetyl-1-pyrroline (2AP) and the structural homologues 6-acetyl-2,3,4,5-tetrahydropyrdine (ATHP, along with its tautomer 6-acetyl-1,2,3,4-tetrahydropyridine), and 2-propionyl-1-pyrroline (2PP) are well-known primary odorants in a variety of foods, such as aromatic rice, popcorn, and breads. However, due to the highly unstable nature of these compounds, the perceivable odors are limited to just few hours after the initial preparation of the foods. The current study presents a novel stabilization method for these potent odorants in dry powder form. Coordination of 2AP, ATHP, and 2PP to zinc ions (ZnI₂, ZnBr₂ or ZnCl₂) resulted in the formation in high yields of stable crystalline complexes, which upon hydration release the free odorant. Heterocyclic nitrogen and carbonyl oxygen atoms are possible binding sites to the zinc ion as indicated infrared spectroscopy. Stability studies showed that 2AP zinc iodide complex is stable at ambient temperature (only 6% reduction after 3 months of storage at 25°C), which may enable its use as a flavoring agent. Meanwhile, the ATHP-ZnI₂ complex was similarly stable and showed over 88% retention after 2 months of storage.

The stability of 2AP complex as flavoring is mainly affected by temperature, moisture, and other possible food matrix components. Therefore, in order to maintain/increase the storage stability, an appropriate package or encapsulation
technology, such as dispersion in an edible oil or lipid or wax encapsulation is suggested to protect the complex from moisture and interactions with other food components. In order to facilitate 2AP complex in foods for flavoring applications, zinc chloride may be preferred since its GRAS status. This novel stabilization process, hopefully, can enable the practical use of the labile, yet powerful flavor compounds 2-acetyl-1-pyrroline and its homologues as flavoring agents.
APPENDIX A Response Factors of Heterocyclics

Response Factor of 2,3-Diethylpyrazine Isotope

Standard: $^{[2]}H_{10}$-2,6-diethylpyrazine 2,3-diethylpyrazine
Mfg/Reference: synthesized Sigma-Aldrich
Purity (GC-FID): 93.4% 98%

Mass Spectra:

Standard Curve:

<table>
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<tr>
<th>Level</th>
<th>Isotope mass ratio</th>
<th>Unlabel mass ratio</th>
<th>Isotope</th>
<th>Unlabel</th>
</tr>
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<tr>
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</tr>
</tbody>
</table>

y = 1.0684x + 0.1898
R² = 0.9926

2,3-diethyl pyrazine

Slope=1.0684
Response Factor = 0.936
Response Factor of 2,6-Diethylpyrazine Isotope

Standard: $[^2\text{H}_{10}]$-2,6-diethylpyrazine
Mfg/Reference: synthesized
Purity (GC-FID): 93.4%

Mass Spectra:

Standard Curve:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concn ug/mL</th>
<th>Mass Ratio 136</th>
<th>Mass Ratio 146</th>
<th>Unlabel</th>
<th>Isotope</th>
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<tr>
<td>26DEP</td>
<td>875</td>
<td>8.3652008</td>
<td>14311635</td>
<td>11988716</td>
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<td>26DEP-d10</td>
<td>1046</td>
<td>2.5095602</td>
<td>203734697</td>
<td>16864253</td>
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<tr>
<td>level 1</td>
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<td>0.8365201</td>
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<tr>
<td>level 2</td>
<td>(3/1)</td>
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<td>36326189</td>
<td>142445638</td>
</tr>
<tr>
<td>level 5</td>
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<td>0.083652</td>
<td>22431974</td>
<td>142445638</td>
<td></td>
</tr>
</tbody>
</table>

\[ y = 1.4404x + 0.0405 \]
\[ R^2 = 1 \]

Slope=1.4404
Response Factor = 0.694
Response Factor of 2-Vinyl-3,6-dimethylpyrazine Isotope

<table>
<thead>
<tr>
<th>Standard:</th>
<th>Isotope</th>
<th>Unlabel</th>
</tr>
</thead>
<tbody>
<tr>
<td>[( \text{H}_2 )]-2-ethyl-3,6-dimethylpyraizine</td>
<td>[( \text{H}_2 )]-2-ethyl-3,6-dimethylpyraizine</td>
<td></td>
</tr>
<tr>
<td>Mfg/Reference:</td>
<td>synthesized</td>
<td>synthesized</td>
</tr>
<tr>
<td>Purity (GC-FID):</td>
<td>91.6%</td>
<td>94.4%</td>
</tr>
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</table>

Mass Spectra:

![Mass Spectra](image)

Standard Curve:

<table>
<thead>
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<th>Mass Ratio</th>
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</tr>
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<td>1/10</td>
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</tbody>
</table>

Slope = 0.6477
Response Factor = 1.544
Response Factor of 2-methylpyrazine Isotope

Standard: $[^2H_3]$-2-methylpyrazine

Mfg/Reference: synthesized

Purity (GC-FID): 86.2%

Mass Spectra:

**Isotope**

**Unlabel**

**Mass Spectra:**

**Standard Curve:**

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<thead>
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<th>Level</th>
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<td>2</td>
<td>3/1</td>
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<td>3</td>
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<td>4</td>
<td>1/3</td>
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<td>24553898</td>
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<tr>
<td>5</td>
<td>1/10</td>
<td>0.11211499</td>
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</tr>
</tbody>
</table>

\[ y = 0.987x - 0.023 \]
\[ R^2 = 0.9998 \]

Slope=0.987

Response Factor = 1.013
Response Factor of 2-Ethylpyrazine Isotope

Standard: [\textsuperscript{12}H\textsubscript{5}]2-ethylpyrazine
Mfg/Reference: synthesized
Purity (GC-FID): 96.3%
Mass Spectra:

<table>
<thead>
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<th>Mass Ratio</th>
<th>Unlabeled</th>
<th>Isotope</th>
</tr>
</thead>
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<td>20,268,3504</td>
</tr>
<tr>
<td>2</td>
<td>3/1</td>
<td>4,916,744,621</td>
<td>58,768,320</td>
</tr>
<tr>
<td>3</td>
<td>1/1</td>
<td>1,638,914,874</td>
<td>17,550,001</td>
</tr>
<tr>
<td>4</td>
<td>1/3</td>
<td>0,546,804,958</td>
<td>17,778,635</td>
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<tr>
<td>5</td>
<td>1/10</td>
<td>0,163,891,487</td>
<td>20,830,717</td>
</tr>
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</table>

Area Ratio vs Mass Ratio

\[ y = 0.6603x - 0.0176 \]
\[ R^2 = 0.9998 \]

Mass Spectra:

\begin{align*}
\text{Standard Curve:} \\
\text{EP} & \quad 1752 \text{ ug/mL} \\
\text{EP-d5} & \quad 1069 \text{ ug/mL} \\
\text{Level} & \quad \text{Mass Ratio} & \quad \text{Unlabeled} & \quad \text{Isotope} \\
1 & \quad 10/1 & \quad 16,389,148,74 & \quad 20,268,3504 & \quad 18,798,218 \\
2 & \quad 3/1 & \quad 4,916,744,621 & \quad 58,768,320 & \quad 17,643,472 \\
3 & \quad 1/1 & \quad 1,638,914,874 & \quad 17,550,001 & \quad 18,000,470 \\
4 & \quad 1/3 & \quad 0,546,804,958 & \quad 17,778,635 & \quad 53,885,951 \\
5 & \quad 1/10 & \quad 0,163,891,487 & \quad 20,830,717 & \quad 18,495,284 \\
\end{align*}

Slope: 0.6603
Response Factor = 1.514
Response Factor of 2,3-Diethyl-5-methylpyrazine Isotope

Standard: 2,3-diethyl-5-[\text{H}_3]-methylpyrazine
Mfg/Reference: synthesized
Purity (GC-FID): 91.2%

Isotope

Unlabel

Mass Spectra:

Standard Curve:

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<tr>
<th>Level</th>
<th>Mass Ratio</th>
<th>Mass Ratio</th>
<th>Mass Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10/1</td>
<td>11.62192394</td>
<td>234833784</td>
</tr>
<tr>
<td>2</td>
<td>3/1</td>
<td>3.486577181</td>
<td>70741592</td>
</tr>
<tr>
<td>3</td>
<td>1/1</td>
<td>1.162192394</td>
<td>21462594</td>
</tr>
<tr>
<td>4</td>
<td>1/3</td>
<td>0.387397465</td>
<td>22810668</td>
</tr>
<tr>
<td>5</td>
<td>1/10</td>
<td>0.116219239</td>
<td>28629845</td>
</tr>
</tbody>
</table>

Slope=0.9188
Response Factor = 1.088
Response Factor of 2,3-Dimethylpyrazine Isotope

Standard: $[^{2}H_3]$-2,3-dimethylpyrazine
Mfg/Reference: synthesized
Purity (GC-FID): 80.6% (with $[^{2}H_3]$-2,6-dimethylpyrazine, 16.5%)

Mass Spectra:

Standard Curve:

<table>
<thead>
<tr>
<th>Mass Ratio</th>
<th>Unlabel</th>
<th>Isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td>level 1</td>
<td>10/1</td>
<td>11.15649606 338979818 33768596</td>
</tr>
<tr>
<td>level 2</td>
<td>5/1</td>
<td>5.578248031 204271038 31356236</td>
</tr>
<tr>
<td>level 3</td>
<td>1/1</td>
<td>1.115649606 39399589 28308169</td>
</tr>
<tr>
<td>level 4</td>
<td>1/5</td>
<td>0.223129921 45999622 146241581</td>
</tr>
<tr>
<td>level 5</td>
<td>1/10</td>
<td>0.111564961 48397985 256507026</td>
</tr>
</tbody>
</table>

\[ y = 0.9116x + 0.3663 \]
\[ R^2 = 0.9807 \]

Slope=0.9116
Response Factor = 1.097
Response Factor of 2,6-Dimethylpyrazine Isotope

Standard: $[^2_2]H_3$-2,6-dimethylpyrazine
Mfg/Reference: synthesized
Purity (GC-FID): 16.5% (with $[^3_3]H_3$-2,3-dimethylpyrazine, 80.6%)

Mass Spectra:

Mass Ratio

<table>
<thead>
<tr>
<th>Level</th>
<th>Mass Ratio</th>
<th>Isotope</th>
<th>Unlabel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10/1</td>
<td>3249821045</td>
<td>272299881</td>
</tr>
<tr>
<td>2</td>
<td>5/1</td>
<td>1624910523</td>
<td>159768077</td>
</tr>
<tr>
<td>3</td>
<td>1/1</td>
<td>3249821045</td>
<td>29149201.5</td>
</tr>
<tr>
<td>4</td>
<td>1/5</td>
<td>0.649964209</td>
<td>34372482</td>
</tr>
<tr>
<td>5</td>
<td>1/10</td>
<td>0.324982105</td>
<td>36829640.5</td>
</tr>
</tbody>
</table>

Area Ratio

\[ y = 0.7895x + 0.697 \]

\[ R^2 = 0.986 \]

Slope = 0.7895
Response Factor = 1.267
Response Factor of 2,5-Dimethylpyrazine Isotope

Standard:  \[^2\mathrm{H}_3\]-2,6-dimethylpyrazine

Mfg/Reference: synthesized

Purity (GC-FID): 16.5% (with \[^2\mathrm{H}_3\]-2,3-dimethylpyrazine, 80.6%)

Mfg/Reference: Sigma-Aldrich

Purity (GC-FID): 98%

Mass Spectra:

Standard Curve:

<table>
<thead>
<tr>
<th>Mass Ratio</th>
<th>25DMP</th>
<th>Unlabel</th>
<th>Isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td>level 1</td>
<td>10/1</td>
<td>5.917442138</td>
<td>87459934</td>
</tr>
<tr>
<td>level 2</td>
<td>5/1</td>
<td>2.958721069</td>
<td>46941576</td>
</tr>
<tr>
<td>level 3</td>
<td>1/1</td>
<td>0.591744214</td>
<td>7895031.5</td>
</tr>
<tr>
<td>level 4</td>
<td>1/5</td>
<td>0.118348843</td>
<td>9120404.5</td>
</tr>
<tr>
<td>level 5</td>
<td>1/10</td>
<td>0.059174421</td>
<td>9602353.5</td>
</tr>
</tbody>
</table>

Area Ratio vs. Mass Ratio graph:

\[ y = 1.3908x + 0.1029 \]

\[ R^2 = 0.995 \]

Slope = 1.3908  
Response Factor = 0.719
Response Factor of 2-Ethyl-3-methylpyrazine Isotope

Standard: \(^{[2}H_5\)-2-ethyl-3-methylpyrazine

Mfg/Reference: synthesized

Purity (GC-FID): 88.7% (with \(^{[3}H_3\)-2-ethyl-6-methylpyrazine, 9.5%)

Mass Spectra:

- **Isotope**
- **Unlabel**

**Standard Curve:**

<table>
<thead>
<tr>
<th>Mass Ratio</th>
<th>ug/mL</th>
<th>Isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td>2E6MP</td>
<td>670</td>
<td>102387623</td>
</tr>
<tr>
<td>2E6MP-d5</td>
<td>495</td>
<td>4954463.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Level</th>
<th>Mass Ratio</th>
<th>2E6MP</th>
<th>2E6MP-d5</th>
<th>Mass Ratio</th>
<th>Isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10/1</td>
<td>13.53535354</td>
<td>102387623</td>
<td>4845463.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5/1</td>
<td>6.767676768</td>
<td>56416404</td>
<td>4540798</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1/1</td>
<td>1.353535354</td>
<td>9792222.5</td>
<td>4082128.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1/5</td>
<td>0.270707071</td>
<td>11318174</td>
<td>23261050</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1/10</td>
<td>0.135353535</td>
<td>12009236</td>
<td>45725253</td>
<td></td>
</tr>
</tbody>
</table>

\[ y = 1.5837x + 0.3523 \]
\[ R^2 = 0.9927 \]

**Response Factor = 0.6314**
Response Factor of 2-Ethyl-6-methylpyrazine Isotope

Standard: 2H\textsubscript{5}-2-ethyl-6-methylpyrazine

Mfg/Reference: synthesized

Purity (GC-FID): 9.5% (with 2H\textsubscript{5}-2-ethyl-3-methylpyrazine, 88.7%)

2-ethyl-6-methylpyrazine
Sigma-Aldrich
55.48% (with 2-ethyl-5-methyl, 42.52%)

Mass Spectra:

Standard Curve:

<table>
<thead>
<tr>
<th>Mass Ratio</th>
<th>Isotope</th>
<th>Unlabel</th>
</tr>
</thead>
<tbody>
<tr>
<td>level 1</td>
<td>10/1</td>
<td>257967605</td>
</tr>
<tr>
<td>level 2</td>
<td>5/1</td>
<td>38967190.5</td>
</tr>
<tr>
<td>level 3</td>
<td>1/1</td>
<td>34662477.5</td>
</tr>
<tr>
<td>level 4</td>
<td>1/5</td>
<td>174835459</td>
</tr>
<tr>
<td>level 5</td>
<td>1/10</td>
<td>308661517</td>
</tr>
</tbody>
</table>

\[ y = 1.3908x + 0.1029 \]
\[ R^2 = 0.995 \]

Area Ratio

Mass Ratio

Slope=0.8706

Response Factor = 1.1489

y = 1.3908x + 0.1029

104
Response Factor of 2-Ethyl-5-methylpyrazine Isotope

Standard:  
- Isotope: $[^3]H_5$-2-ethyl-6-methylpyrazine  
- Unlabel: 2-ethyl-5-methylpyrazine

Mfg/Reference:  
- synthesized  
- Sigma-Aldrich

Purity (GC-FID):  
9.5% (with $[^3]H_5$-2-ethyl-3-methylpyrazine, 88.7%)  
42.52% (with 2-ethyl-6-methyl, 55.48%)

Mass Spectra:

Standard Curve:

<table>
<thead>
<tr>
<th>Level</th>
<th>Mass Ratio</th>
<th>Unlabel</th>
<th>Isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10/1</td>
<td>18.36363636</td>
<td>76401654.5</td>
</tr>
<tr>
<td>2</td>
<td>5/1</td>
<td>9.181818182</td>
<td>41303099</td>
</tr>
<tr>
<td>3</td>
<td>1/1</td>
<td>1.836363636</td>
<td>7085711.5</td>
</tr>
<tr>
<td>4</td>
<td>1/5</td>
<td>0.367272727</td>
<td>8160581.5</td>
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<tr>
<td>5</td>
<td>1/10</td>
<td>0.183636364</td>
<td>8551580</td>
</tr>
</tbody>
</table>

Area Ratio vs. Mass Ratio:

\[
y = 0.8702x + 0.218 \\
R^2 = 0.9944 \\
\]

Slope = 0.8702  
Response Factor = 1.1491
Response Factor of 2-Ethyl-3,6-diethylpyrazine Isotope

Standard: $[^2H_5]$-2-ethyl-3,6-dimethylpyrazine
Mfg/Reference: synthesized
Purity (GC-FID): 98%
Mass Spectra:

Isotope

Unlabel

Standard Curve:

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Mass Spectra (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2E36DMP</td>
<td>886</td>
</tr>
<tr>
<td>2E36DMP-d5</td>
<td>1048</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mass Ratio</th>
<th>Unlabel</th>
<th>Isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/1</td>
<td>8.454198473</td>
<td>138285817</td>
</tr>
<tr>
<td>5/1</td>
<td>4.227099237</td>
<td>76970993.5</td>
</tr>
<tr>
<td>1/1</td>
<td>0.845419847</td>
<td>13443371</td>
</tr>
<tr>
<td>1/5</td>
<td>0.169083969</td>
<td>16003051.5</td>
</tr>
<tr>
<td>1/10</td>
<td>0.084541985</td>
<td>16957180</td>
</tr>
</tbody>
</table>

Slope = 0.926
Response Factor = 1.080
Response Factor of 2-Ethyl-3,5-diethylpyrazine Isotope

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Unlabel</th>
</tr>
</thead>
<tbody>
<tr>
<td>[(^2\text{H}_5)]-2-ethyl-3,5-dimethylpyrazine</td>
<td>2-ethyl-3,5-dimethylpyrazine</td>
</tr>
<tr>
<td>Mfg/Reference:</td>
<td>synthesized</td>
</tr>
<tr>
<td>Purity (GC-FID):</td>
<td>91.6%</td>
</tr>
</tbody>
</table>

**Mass Spectra:**

![Mass Spectra Graph](Image)

**Standard Curve:**

<table>
<thead>
<tr>
<th>Mass Ratio</th>
<th>Unlabeled</th>
<th>Isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td>level 1</td>
<td>10/1</td>
<td>11.97578902</td>
</tr>
<tr>
<td>level 2</td>
<td>5/1</td>
<td>5.987894509</td>
</tr>
<tr>
<td>level 3</td>
<td>1/1</td>
<td>1.197578902</td>
</tr>
<tr>
<td>level 4</td>
<td>1/5</td>
<td>0.23951578</td>
</tr>
<tr>
<td>level 5</td>
<td>1/10</td>
<td>0.11975789</td>
</tr>
</tbody>
</table>

\[ y = 1.0951x + 0.1489 \]
\[ R^2 = 0.9961 \]

Slope = 1.0951
Response Factor = 0.913
Response Factor of Trimethylpyrazine Isotope

Standard: 2,3-[^3H]_3.5-trimethylpyrazine Isotope
Mfg/Reference: synthesized Sigma-Aldrich
Purity (GC-FID): 96.6% 98%

Mass Spectra:

Standard Curve:

<table>
<thead>
<tr>
<th>Level</th>
<th>Mass Ratio</th>
<th>TMP ug/mL</th>
<th>TMP-d3 ug/mL</th>
<th>Unlabel</th>
<th>Isotope</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>11.63179916</td>
<td>299930415</td>
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</tr>
<tr>
<td>2</td>
<td>3/1</td>
<td>3.489539749</td>
<td>89027912</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>1/1</td>
<td>1.163179916</td>
<td>26338039</td>
<td>25475066</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1/3</td>
<td>0.387726639</td>
<td>27071926</td>
<td>78163821</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1/10</td>
<td>0.116317992</td>
<td>32042752</td>
<td>26046207</td>
<td></td>
</tr>
</tbody>
</table>

Area Ratio  

\[ y = 0.9354x + 0.0199 \]
\[ R^2 = 0.9996 \]

Response Factor = 1.069
**Response Factor of 2-Acetyl-1-pyrroline (GC-FID)**

<table>
<thead>
<tr>
<th>Target</th>
<th>Internal standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-acetyl-1-pyrroline</td>
<td>collidine</td>
</tr>
<tr>
<td>synthesized</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>91.0%</td>
<td>98%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cncl of 2AP (ug/mL)</th>
<th>Cncl of I.S. (ug/mL)</th>
<th>Area of 2AP (RT=7.898)</th>
<th>Area of I.S. (RT=10.495)</th>
<th>Area ratio</th>
<th>cncl ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>4384</td>
<td>804</td>
<td>96499</td>
<td>51255</td>
<td>1.8827</td>
<td>5.4532</td>
</tr>
<tr>
<td>2192</td>
<td>804</td>
<td>48435</td>
<td>50580</td>
<td>0.9576</td>
<td>2.7266</td>
</tr>
<tr>
<td>1096</td>
<td>804</td>
<td>25470</td>
<td>52179</td>
<td>0.4881</td>
<td>1.3633</td>
</tr>
<tr>
<td>548</td>
<td>804</td>
<td>10979</td>
<td>44946</td>
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<td>0.6817</td>
</tr>
<tr>
<td>274</td>
<td>804</td>
<td>5435</td>
<td>48005</td>
<td>0.1132</td>
<td>0.3408</td>
</tr>
</tbody>
</table>

\[ y = 0.3448x + 0.0086 \]
\[ R^2 = 0.9998 \]

Slope = 0.3448

Response Factor = 2.8998

a. 2-Acetylpyrroline = 4384 ug/mL in phosphate buffer (50 mM pH=7.1).
b. Internal standard (I.S.) = 804 ug/mL (methylene chloride)
c. 2-Acetyl-1-pyrroline solution (water) 0.5 mL + I.S. 0.5 mL, vortex 1 min, then centrifuge (3000 rpm × 1 min). 1 uL solvent was injected into GC-FID.
Response Factor of 2-Acetyl-1-pyrroline (GC-MS)

<table>
<thead>
<tr>
<th>Ccn of 2AP (ug/mL)</th>
<th>Ccn of I.S. (ug/mL)</th>
<th>Area of 2AP (ug/mL)</th>
<th>Area of I.S. (ug/mL)</th>
<th>area ratio</th>
<th>cnen ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>3713</td>
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<td>80948036</td>
<td>81208945</td>
<td>0.9968</td>
<td>3.4475</td>
</tr>
<tr>
<td>1856.5</td>
<td>1077</td>
<td>51932978</td>
<td>101746461</td>
<td>0.5104</td>
<td>1.7238</td>
</tr>
<tr>
<td>928.25</td>
<td>1077</td>
<td>26508838</td>
<td>10645065</td>
<td>0.2489</td>
<td>0.8619</td>
</tr>
<tr>
<td>464.125</td>
<td>1077</td>
<td>8301081</td>
<td>88199866</td>
<td>0.0941</td>
<td>0.4309</td>
</tr>
<tr>
<td>232.0625</td>
<td>1077</td>
<td>3301794</td>
<td>86415427</td>
<td>0.0382</td>
<td>0.2155</td>
</tr>
</tbody>
</table>

\[ y = 0.2977x - 0.02 \]
\[ R^2 = 0.9987 \]

Slope = 0.2977
Response Factor = 3.3546

a. 2-Acetylpyrroline in phosphate buffer (50 mM, pH=7.1).
b. Internal standard (I.S.) collidine = 27.2 mg in 25 mL methylene chloride * 99% (purity) = 1077ug/mL.
c. 2-Acetyl-1-pyrroline solution 0.5 mL + I.S. 0.5 mL, vortex 1 min. then centrifuge (3000 rpm × 1 min). 1 uL solvent was injected into GC-MS.
APPENDIX B NMR Spectra of Deuterated Alkylpyrazines

$^1$H NMR Spectra of Labeled and Unlabeled Methylpyrazine

$[\text{H}_3]\text{-Methylpyrazine}$

Methylpyrazine
$^1$H NMR Spectra of Labeled and Unlabeled Ethylpyrazine

$[^1\text{H}_5]$-Ethylpyrazine

Ethylpyrazine
$^1$H NMR Spectra of Labeled and Unlabeled Dimethylpyrazine

Mixture of $[^1H]_3$-2,3(or 6)-dimethylpyrazines

2,3-Dimethylpyrazines

2,6-Dimethylpyrazines
$^1$H NMR Spectra of Labeled and Unlabeled 2,6-Dimethylpyrazine

[$^1$H$_3$]-2,$[^1$H$_3$]-6-Dimethylpyrazines
$^1$H NMR Spectra of Labeled and Unlabeled Trimethylpyrazine

$[^1\text{H}_3]$-Trimethylpyrazines

Trimethylpyrazines
$^1$H NMR Spectra of Labeled and Unlabeled 2-Ethyl-3(or 6)-methylpyrazine

$[^{1}H_5]$-2-Ethyl-3(or 6)-methylpyrazines

2-Ethyl-3-methylpyrazines
$^1$H NMR Spectra of Labeled and Unlabeled Ethyl-dimethylpyrazine

$[^1H_5]$-2-Ethyl-3,5-dimethylpyrazines

2-Ethyl-3,6-dimethylpyrazines
$^1$H NMR Spectra of Labeled and Unlabeled 2,6-Diethylpyrazines

$[^1\text{H}_3]2,[^1\text{H}_3]6$-Diethylpyrazines

2,6-Diethylpyrazines

STANDARD PROTON PARAMETERS

impurity
$^1$H NMR Spectra of Labeled and Unlabeled 2,3-Diethyl-5-methylpyrazines

2,3-Diethyl-$[^1]$H$_3$-5-methylpyrazines

2,3-Ethyl-5-methylpyrazines
$^1$H NMR Spectra of 2-Vinyl-3,6-dimethylpyrazines

2-Vinyl-3,6-dimethylpyrazines
APPENDIX C IR Spectra of Heterocyclic Complexes

IR Spectra of 2-Acetyl-1-pyrroline and its Complexes

![IR Spectra Graph]

- 2-Acetyl-1-pyrazine
- 2-Acetyl-1-pyrazine Zinc iodide complex
- 2-Acetyl-1-pyrazine Zinc bromide complex
- 2-Acetyl-1-pyrazine Zinc chloride complex
IR Spectra of ATHPs (6-acetyl-2,3,4,5-tetrahydropyridine, 6-acetyl-1,2,3,4-tetrahydropyridine) and Their Complexes

- ATHPs
- ATHPs, Zinc iodide complex
- ATHPs, Zinc bromide complex
- ATHPs, Zinc chloride complex
IR Spectra of 2-Propionyl-1-pyrroline and its Complexes

2-Propionyl-1-pyrroline
2-Propionyl-1-pyrroline Zinc iodide complex
2-Propionyl-1-pyrroline Zinc bromide complex
2-Propionyl-1-pyrroline Zinc chloride complex
IR Spectra of 2-Acetyl-2-thiazoline and its Complexes

- 2-Acetyl-2-thiazoline
- 2-Acetyl-2-thiazoline Zinc iodide complex
- 2-Acetyl-2-thiazoline Zinc bromide complex
- 2-Acetyl-2-thiazoline Zinc chloride complex
IR Spectra of 2-Acetylpyridine and its Complexes

2-Acetylpyridine

Zinc iodide complex

Zinc bromide complex

Zinc chloride complex
IR Spectra of 2-Acetylpyrazine and its Complexes
IR Spectra of 2-Acetylthiazole and its Complexes

- 2-Acetylthiazole
- 2-Acetylthiazole Zinc iodide complex
- 2-Acetylthiazole Zinc bromide complex
- 2-Acetylthiazole Zinc chloride complex
APPENDIX D Synthesis Procedures of 2AP and ATHP(S)

Synthesis of 2-Acetyl-1-pyrroline (1-(3,4-dihydro-2H-pyrrol-5-yl)ethanone)

- Pyrrolidine, 99% (Sigma-Aldrich P73803-100ML; FW=71.12)
- Silver nitrate, (Fisher S181-25; FW=169.87)
- Sodium persulfate, ≥ 98% (Sigma-Aldrich 216232-500G; FW=238.1)
- Potassium cyanide, ≥ 96% (Sigma-Aldrich 207810-25G; FW=65.12)
- tert-Butanol, ≥ 99.0% (Sigma-Aldrich 360538-500ML; FW=74.12)
- Triethylamine, ≥ 99% (Sigma-Aldrich T0886-1L; FW=101.19, d=0.726)
- Iodomethane, ≥ 99% (Sigma-Aldrich T0886-1L; FW=141.94, d=2.28)
- Magnesium, turnings, 99.8% metals basis (Sigma-Aldrich 403148-50G; FW=24.31)
- DCM – dichloromethane (methylen chloride)

Pyrrolidine trimer
1) Placed 7g (174 mmol) NaOH in a 250 mL-round-bottom flask with 50 mL water under water/ice bath (temperature below 10°C)
2) 6.24g pyrrolidine (87 mmol) was added and then 73 mg silver nitrate (0.17 mmol; 0.2% mol as catalye) was added
3) 20.7g (87 mmol) sodium persulfate in 50 mL water was added dropwise (by a separatory funnyl) into above solution (below 10°C, adding took 20 min). After adding, this mixture was stirred for 2.5h allowed at ambient temperature
4) Reaction mixture (if form some solid salt, filtered) was extracted with DCM (20 mL × 3), and then dried over sodium sulfate. Solvent was removed at 40°C under vacuum to obtain pyrrolidine trimer as orange oil (yield= 3.2g, 45 mmol, 51.3%). Kept at -20°C

Cyanopyrrolidine
1) Pyrrolidine trimer 3.2g (45 mmol) was added of potassium cyanide 3.9g (60 mmol; 150% mmol) in 20 mL water at ambient temperature
2) HCl (4N) 15 mL (60 mmol) was added dropwise through a separatory funnyl
3) Additional HCl was added in order to acidify this solution to about pH = 4.0
4) Reaction mixture was stirred at ambient temperature for 2h (longer may better)
5) Reaction was alkalized (pH>8) by NaOH pallet (0.6g plus), and then extracted with DCM (20 mL × 3)
6) Solvent layers were combined and then dried over sodium sulfate
7) Solvent was concentrated into about 20 mL, then HVT
8) During HVT, heating under boiling water was needed for the final 20 min
9) Distillate was dried and solvent was removed (40°C, vacuum) to obtained cyanopyrrolidine 2g (21 mmol)
2-Cyano-1-pyrroline (FW = 94)

Step 1. Preparation of tert-butyhypochlorite (t-BuOCl)
1) A 1 L Erlenmeyer flask was added of 300 mL commercial bleach solution (~7% chlorine), a stir bar (stirred at high speed), 10 mL acetic acid, and 15 mL tert-butanol
2) Mixture was Stirred at ice bath (0°C) for 60 min (turn light off)
3) Solution was transferred into a separatory funnel (t-BuOCl is a yellow oil-like liquid on the top of this solution)
4) Drained away down layer, and then washed with 10% sodium carbonate (20 mL x 2), then water (10 mL x2)
5) t-BuOCl was collected and dry over sodium sulfate for 2h, then transferred to a vial with sodium sulfate for further drying (purity should over 90%)
6) Kept at -20°C, wrapped with aluminum foil

Step 2. Titration of t-BuOCl

- Reagent: 2.48g Na$_2$S$_2$O$_3$•5H$_2$O + 10mg Na$_2$CO$_3$ in 100 mL water (brown flask)

Procedure:
1) 0.5g KI in 250 mL Erlenmeyer flask with stir bar was added of water (100 mL)
2) t-BuOCl about 0.1g (weight out and recorded) was added, and then 4 mL acetic acid was added
3) Added of a little bit starch
4) Titrated by 0.1M Na$_2$S$_2$O$_3$ solution (about 20 mL was needed for a 100% t-BuOCl solution)
5) Calculated through the flowing formula:
   \[ \text{mL of Na}_2\text{S}_2\text{O}_3 \text{solution} / 20 = \text{mMole tBuOCl} \]
   Or Purity % = \[ \frac{\text{mL}}{\text{weight mg}} \times 5.4285 \times 100\% \]

Step 3. Chlorination and Oxidation
1) Cyanopyrrolidine 21 mmol was diluted in ether (30 -50 mL) in a 200 mL-round-bottom flask equipped with a stir bar and nitrogen purge (moisture sensitive)
2) t-BuOCl 21 mmol was added dropwise in 1 min under ice bath and then stirred for 30 min
3) TEA 21 mmol was added, and then the reaction mixture was allowed to stir at ambient temperature for at least 4 h or overnight
4) Significant precipitate formed. Centrifuged, solvent was collected, and the residue was washed with fresh ether, and then centrifuged.
5) Solvent layers were combined and then removed at 40°C on vacuum
6) Residue was titrated with fresh ether, and then centrifuged
7) Solvent was recovered, and removed at 40°C on vacuum. Black oil-like stuff was obtained
8) Purification was conducted via silica gel chromatography
• 10g silica gel was conditioned through a solvent mixture of 20% ether and 80% pentane (E/P = 2/8)
• After loading, elute with 50 mL solvent (E/P = 2/8), monitored with GC
• Elute with 50 ~ 100 mL solvent (E/P = 5/5), monitored with GC
• Fractions with target compound were collected then concentrated into about 20 mL, and then HVT

9) During HVT, heated at 70°C for the final 20 min
10) Distillate was collected, dried, and then solvent was removed to obtained pure 2-cyano-1-pyrroline 1g, purity = 99.5%
11) Mass spectra of 2-cyano-1-pyrroline for your reference

2-acetyl-1-pyrroline
1) To a 100-mL three-neck round bottom flask equipped with a stir bar, 371 mg magnesium (16 mmol), 20 mL ether, iodine (a bit), and ~ 1mL iodomethane were added under ice bath with good stirring and nitrogen purge
2) 2-Cyano-1-pyrroline was added dropwise and the reaction mixture was stirred for 30 min
3) 50 mL 10% NH₄Cl water solution was added to quench the reaction, and then extracted with ether (20 mL ×3)
4) Solvent layer was combined and then dried over sodium sulfate
5) Solvent was concentrated into 20 mL, and then HVT
6) Distillate was dried over sodium sulfate and then solvent was removed
7) 0.6 g was obtained, purity = 91%

Reference:

Synthesis of 6-Acetyl-2,3,4,5-tetrahydropyridine and 6-Acetyl-1,2,3,4-tetrahydropyridine

- Sodium methoxide, 25 wt. % in methanol (4.37M)(Sigma-Aldrich 156256-1L; FW=54.02)
- Piperidine, 99% (Sigma-Aldrich 104094-100ML; FW= 85.15)
- Triethylamine, ≥ 99% (Sigma-Aldrich T0886-1L; FW=101.19, d=0.726 )
- Iodomethane, ≥ 99.0% (Sigma-Aldrich 67692-100ML; FW=141.94, d=2.28)
- Magnesium, turnings, 99.8% metals basis (Sigma-Aldrich 403148-50G; FW=24.31)
- tert-Butylhypochlorite – t-BuOCl
- DCM – dichloromethane (methylene chloride)

Triperidine
1) Placed 10g (117 mmol) piperidine in a 250 mL-round-bottom flask with 60 mL ether under ice bath with good stirring and nitrogen purge
2) t-BuOCl 117 mmol was added dropwise in 1 min. The reaction mixture was stirred for 30 min under ice bath
3) Solvent was evaporated on vacuum at 20°C to about 10 mL left
4) Sodium methoxide 35 mL (130% mol) was diluted with 35 mL methanol and then added
5) Reaction mixture was stirred at ambient temperature for 20 min and then reflux for 45 min
6) Precipitate was filtered, washed with methanol (this step differ from ref. 1)
7) Filtrates was collected, the solvent (methanol) was evaporated (this step differ from ref. 1)
8) Diethyl ether 50 mL was added into the residue, and then filtered (this step differ from ref. 1)
9) Filtrate was collected and then washed with 1N NaOH solution (30 mL) (this step differ from ref. 1)
10) Solvent was dried over sodium sulfate and then removed at 40°C on vacuum to obtained 6g triperidine (this step differ from ref. 1)

Cyanopiperidine (FW=110)
1) Triperidine 6g was added of potassium cyanide 6.6 g in 25 mL water at ambient temperature.
2) HCl (4N) 25 mL was added dropwise through a separatory funnyl
3) Additional HCl was added in order to acidify this solution to about pH = 4.5
4) Reaction mixture was stirred at ambient temperature for overnight
5) Reaction was alkalized by NaOH pallet (0.6g plus) to pH = 8.5, and then extracted with DCM (20 mL × 3)
6) Solvent layers were combined and then dried over sodium sulfate
7) Solvent was concentrated into about 20 mL, then HVT
8) During HVT, heating by boiling water bath was needed for the final 20 min
9) Distillate was dried and solvent was removed (40°C, vacuum) to obtained cyanopiperidine 6.7g (impure)

**6-Cyano-2,3,4,5-tetrahydropyridine and 6-cyano-1,2,3,4-tetrahydropyridine (FW=108)**

1) Cyanopiperidine 6.7g was diluted in ether (30 -50 mL) in a 200 mL-round-bottom flask equipped with a stir bar and nitrogen purge (moisture sensitive).
2) t-BuOCl 60 mmol was added dropwise in 1 min under ice bath and then stirred for 30 min
3) TEA 60 mmol was added, and then the reaction mixture was allowed to stir at ambient temperature for at least 4 h or overnight
4) Significant precipitate formed. Centrifuged, solvent was collected, and the residue was washed with fresh ether, and then centrifuged.
5) Solvent layers were combined and then removed at 40°C under vacuum.
6) Residue was titrated with fresh ether, and then centrifuged
7) Solvent was recovered, and removed at 40°C under vacuum. Black oil-like stuff was obtained
8) Purification was conducted via silica gel chromatography
   - 15g silica gel was conditioned through a solvent mixture of 20% ether and 80% pentane (E/P = 2/8)
   - After loading, elute with 50 mL solvent (E/P = 2/8), monitored with GC
   - Elute with 100 mL solvent (E/P = 5/5), monitored with GC
   - Fractions with target compound were collected then concentrated into about 20 mL, and then HVT
9) During HVT, heated at 70°C for the final 20 min
10) Distillate was collected, dried, and then solvent was removed to obtained target compounds 4 g, purity 85% by GC-MS
11) GC-MS (INNOWAX) gave two peaks

12) Mass spectra of 6-cyano-2,3,4,5-tetrahydropyridine (first peak) and 6-cyano-1,2,3,4-tetrahydropyridine (second peak) for your reference
6-Acetyl-2,3,4,5-tetrahydropyridine and 6-Acetyl-1,2,3,4-tetrahydropyridine

1) To a 100-mL three-neck round bottom flask equipped with a stir bar, 1.46 g magnesium (60 mmonl), 50 mL ether, iodine (a bit), and ~ 4mL iodomethane were added under ice bath with good stirring and nitrogen purge (200% Grignard reagent)

2) Cyano-tetrahydropryidine was added dropwise and the reaction mixture was stirred for 30 min

3) 50 mL 10% NH₄Cl water solution was added to quench the reaction, and then extracted with diethyl ether (20 mL ×3)

4) Solvent layer was combined and then dried over sodium sulfate

5) Solvent was concentrated into 20 mL, and then HVT

6) Distillate was dried over sodium sulfate and then solvent was removed

7) Final 0.4 g, purity = 83% (even use 2X Grignard reagent, the starting material still left some)

8) 6-Acetyl-2,3,4,5-tetrahydropyridine, RT=6.4

6-acetyl-1,2,3,4-tetrahydropyridine, RT=7.2

Reference:
