Nitrogen Isotope Analysis of Ammonium and Glycine

Method Development for Aqueous Solutions and Soil Extracts

Elin Norlin
Faculty of Forest Sciences
Department of Forest Ecology
Umeå

Doctoral thesis
Swedish University of Agricultural Sciences
Umeå 2005

Acta Universitatis Agriculturae Sueciae
2005: 84

ISSN 1652-6880
ISBN 91-576-6983-X

Tryck: Arkitektkopia, Umeå 2005

Abstract

Norlin, E., 2005. Nitrogen Isotope Analysis of Ammonium and Glycine. Method Development for Aqueous Solutions and Soil Extracts. Doctor´s dissertation.
ISSN 1652-6880, ISBN 91-576-6983-X

Stable isotope techniques can be used as a tool in nitrogen cycling studies of different ecosystems. The studies are based on measurement of the heavy (^{15}N)- to- light (^{14}N) isotopic ratios of nitrogen in different biospheric pools. Isotope ratio mass spectrometry (IRMS) is the most precise technique to use for analysis of nitrogen isotopic ratios. This thesis deals with the development of methods for compound-specific nitrogen isotope analysis of ammonium and glycine in aqueous solutions and soil extracts using Gas Chromatography - Combustion (GC-C) - IRMS.

For ammonium, three different techniques were developed: equilibrium headspace analysis, solid phase microextraction (SPME) and the purge and trap (P & T) technique, which were all based on conversion of ammonium to ammonia with subsequent separation of ammonia for analysis. In the SPME and P & T approaches, custom-made absorbents were used for pre-concentration, followed by thermal desorption into the GC-C-IRMS system. For the equilibrium headspace technique, high precision measurements of the nitrogen isotopic ratio were obtained for concentrations above 420 mg N L^{-1}. With further improvements and the use of suitable equipment, the method has the potential to be used for solutions containing ammonium in the low mg N L^{-1} range. The SPME technique increased the sensitivity by a factor of ≈ 3 compared to the headspace technique, but was less precise. In addition, the Nafion® material used for absorption showed a memory effect in the desorption step. With the P & T technique a large variation in the measured isotopic value was observed (using solutions containing 2 mg N L^{-1}) which was due to a non-quantitative thermal desorption. However, with further improvements, the P & T technique has the potential to be used for samples containing below 1.0 µg N, which is a much lower amount than that possible with any method used today.

A method for determination of the nitrogen isotopic ratio in free glycine in soil extracts was also developed. By a combination of sample pre-concentration and Isotope Dilution Mass Spectrometry (IDMS), it was possible to determine isotopic ratio in soil extracts with a glycine concentration of only 3 µM (0.042 mg N L^{-1}). The precision obtained was sufficient for use with tracer studies and was higher by an order of magnitude than the precision obtained with conventional GC-MS.

Key words: nitrogen, stable isotope, isotope ratio mass spectrometry, ammonium, glycine, amino acid, solid phase microextraction, purge and trap, headspace analysis.

Author´s adress: Elin Norlin, Department of Forest Ecology, SLU, S-901 83 UMEÅ, Sweden.

Contents

Appendix

Paper I-IV

This doctoral thesis is based upon the following Papers, hereafter referred to by the respective Roman numerals:

I. Norlin, E., Irgum, K. and Ohlsson, K. E. A., 2002. Determination of the $^{15}N/^{14}N$ Ratio of Ammonium and Ammonia in Aqueous Solutions by Equilibrium Headspace-Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry. *The Analyst 127,* 735-740.

II. Norlin, E., Irgum, K. and Ohlsson, K. E. A., 2003. Nitrogen Isotope Analysis of Ammonium in Aqueous Solutions Using a Perflourosulfonated Ionomer Membrane for Solid-Phase Microextraction. *Rapid Communications in Mass Spectrometry 17,* 936-942.

III. Norlin, E., Nguyen, D., Ohlsson, K. E. A. and Irgum, K., 2005. Development of Sorption Tubes with a Sulfonated Styrene-Divinylbenzene Copolymer Surface Layer for Use in Nitrogen Isotope Analysis of Ammonium and Ammonia. Manuscript.

IV. Norlin, E., Irgum, K. and Ohlsson, K. E. A., 2005. Nitrogen Isotope Analysis of Free Glycine in the Mor Layer of a Forest Soil using Isotope Dilution Mass Spectrometry. Submitted manuscript.

Introduction

This thesis deals with the analysis of stable isotopes of nitrogen within glycine and ammonium present in aqueous solutions and soil extracts. In this introduction, terms that are important for further understanding of the thesis are explained.

Stable Isotopes

Two **isotopes** of the same element have the same number of protons and electrons, but differ in the number of neutrons, thus the relative molecular masses are different.

An isotope of an element can be either stable or radioactive. Whereas radioactive isotopes undergo spontaneous decay with time, stable isotopes do not. Only stable isotopes are considered in this thesis.

Natural Abundance

For the elements H, C, N, O and S, there is one dominating stable isotope (the major isotope) and either one or two stable or radioactive isotopes occurring less frequently (minor isotopes). The so called natural abundance of an element is the composition of its isotopes occurring in nature (Table 1). The variations in natural abundance are small, but generally measurable, and depend on processes discriminating against isotopes (see section on Isotopic fractionation).

Table 1. Natural abundance (in %) of the two most abundant stable isotopes of different elements studied by IRMS.

Compound	Major isotope	Abundance (AP)	Minor isotope	Abundance (AP)
Hydrogen	^{1}H	99.98	^{2}H	0.02
Carbon	^{12}C	98.89	^{13}C	1.11
Nitrogen	^{14}N	99.63	^{15}N	0.36
Oxygen	^{16}O	99.76	^{18}O	0.20
Sulphur	^{32}S	95.02	^{34}S	4.22

The isotopic composition of a substance can be expressed in different ways (Slater *et al.*, 2001);

Atom % (AP) = the abundance of an isotope in percent,

$$AP_S = \frac{R_s}{(1+R_s)} \cdot 100 \quad (1)$$

where R is the molar quotient of heavy to light molecule (isotopic ratio) and the subscript s = sample.

Enrichment (Atom % excess, APE) = the isotopic abundance in a sample above a specified background (b) level,

$$APE = AP_S - AP_b \tag{2}$$

δ value = the deviation, in per mil, from the internationally accepted primary reference material for the specific element,

$$\delta(‰) = \left(\frac{R_s}{R_{standard}} - 1 \right) \cdot 1000 \tag{3}$$

The δ notation has been introduced to express very small variations in the natural isotopic composition. For nitrogen, the primary reference material is atmospheric air (R=0.0036765), which is known to have a very stable and evenly distributed isotopic composition around the world (Mariotti, 1983).

Tracer Studies

In tracer studies, an isotopically labelled compound is added to the system studied. The movement and conversion of the compound can then be studied in time and space. The advantage of using an IRMS instrument in such studies is that very small differences in the amount of tracer can be measured. Hence, only a small amount of tracer needs to be added and the disturbance of the natural system can be minimized.

Isotopic Fractionation

Two isotopes of the same element have slightly different physical and chemical properties due to differences in mobility and binding energy of chemical bonds. These differences result in a phenomenon called isotopic fractionation (also called isotopic discrimination) where small, but usually measurable, deviations from natural isotopic abundance can arise in the atom composition of various compounds. Generally, the effects of isotopic fractionation are less pronounced for molecules with higher molecular masses.

There are two types of isotopic fractionation: kinetic and equilibrium (or thermodynamic) fractionation. Kinetic fractionation occurs in one-way processes during transition of a compound into another state or into another compound (Mook, 2002), and is due to different rates of reaction or gaseous diffusion between isotopes. The effect on the isotopic composition of the substrate and the product during a reaction in a closed system is illustrated in Figure 1. The product becomes heavier as the reaction proceeds and at completion the isotopic ratio of the product is identical to the initial isotopic ratio of the reactant.

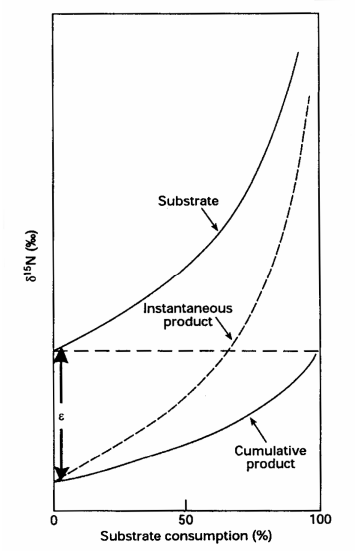

Figure 1. Changes in $\delta^{15}N$ of substrate and product during a reaction in a closed vessel, due to kinetic isotopic fractionation. Reprinted from Högberg (1997) with permission. Copyright 1997, Blackwell Publishing.

The kinetic isotopic fractionation factor (here called α_k to distinguish it from the *equilibrium* fractionation factor), is defined as the ratio between the reaction rates of the light (k_L) and heavy (k_H) isotopes, respectively;

$$\alpha_k = \frac{k_L}{k_H} \tag{4}$$

Equilibrium isotopic fractionation is a two-way process which results in different isotopic ratios for two compounds in chemical or physical equilibrium (Mook, 2002). The equilibrium isotopic fractionation factor, α_{eq}, is defined as:

$$\alpha_{eq} = \frac{R_a}{R_b} \tag{5}$$

where subscripts a and b represent the two compounds in equilibrium.

The difference, or shift, in isotopic composition between substrate (δ_S) and product (δ_P) in a reaction can also be expressed as ε, according to;

$$\varepsilon = \delta_S - \delta_P \tag{6}$$

At isotopic compositions close to natural abundance, a ε value of 5 ‰ corresponds approximately to a fractionation factor α, of 1.005. With a positive ε, the product is said to be enriched compared to the substrate and with a negative ε, the product is depleted.

The likelihood of isotopic fractionation determines sample preparation and other analytical procedures. If the kinetic fractionation during a sample preparation step is large, the reaction, or diffusion process, needs to go to completion in order to obtain a correct estimate of the isotopic ratio. Equilibrium isotopic fractionation is easier to correct for using standards of known isotopic composition (*e.g.* for ^{15}N in ammonia in Paper **I** and ^{18}O in water (Scrimgeour, 1995).

Stable Isotope Analysis using Isotope Ratio Mass Spectrometry

Very accurate and precise measurements of stable isotope ratios of the elements in Table 1 are generally performed with Isotope Ratio Mass Spectrometry (IRMS). The IRMS instruments are all of the magnetic sector type, with an electron impact (EI) ion source prior to the flight tube, in which the ions are separated according to their mass-to-charge ratio (m/z) (Figure 2).

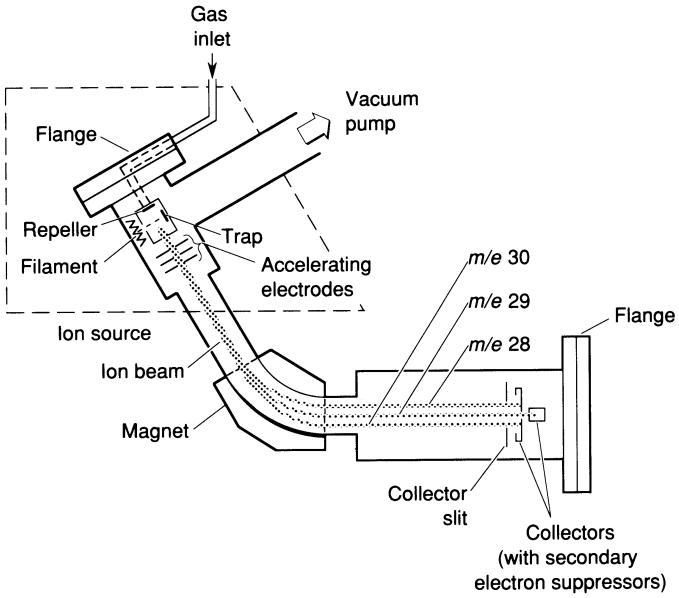

Figure 2. Schematic diagram of an IRMS system for nitrogen isotope analysis. Reprinted from Mulvaney (1993) with permission. Copyright 1993, Elsevier.

Prior to mass spectrometric detection, all compounds are converted to the gaseous state (for example through combustion or pyrolysis), either the preferred elemental gases (N_2 for nitrogen and H_2 for hydrogen), or their gaseous oxides (CO_2 for carbon and oxygen and SO_2 for sulphur).

In comparison to conventional "organic MS", IRMS gives more precise estimates of isotopic ratios due to the total conversion of a sample into simple gas molecules and the simultaneous measurement of isotopes with a multiple collection system. Mass spectrometers for organic compounds, in addition to providing precise determinations of mass numbers, are on the other hand, designed to make fast scans (except Time of Flight (TOF) instruments) over a wide range of mass numbers and hence providing structural information. In Table 2, the principles for IRMS are compared with those of "organic MS".

Hybrid systems, where the carrier gas flow is split between an organic MS and an IRMS, before combustion takes place, provide structural information enabling identification of separated compounds as well as giving their isotopic elemental ratios (Meier-Augenstein *et al.*, 1994; Meier-Augenstein, 1995; Hall *et al.*, 1999).

Table 2. Comparison of a conventional organic GC- MS and a GC-C-IRMS.

	GC-MS	GC-C-IRMS
Type of analyte	Complex organic molecules	Simple gas molecules
Resolution (m/Δm)	Low (100) to high (>10000)	Low (< 100)
Mass separation system	Magnetic sector / quadrupole / time of flight (TOF)/ ion-trap	Magnetic sector
Scanning	Yes, for all except TOF	No
Detection	Single detector (electron multiplier)	Multi-collector (2-3 Faraday cups)
Precision	≈ 0.3 $AP^{15}N$[a)] (850‰)	≈0.00007 $AP^{15}N$ (0.2 ‰) at natural abundance[b)] ≈0.0038 (11 ‰) at 3 $AP^{15}N$ [b)]
Detection limit	0.14-1.4 pg N[a)]	0.01-0.1 µg N

a) from Persson and Näsholm (2001) using an ion trap mass sectrometer.
b) from Prosser (1993)

The limit of detection in IRMS analysis in commonly defined as the amount of the element under consideration required to give an isotopic ratio (expressed as a mean ± standard deviation, SD, of analytical replicates) of specified precision. The development of a new generation of continuous flow (CF) instruments during recent decades, where samples are pre-treated on-line and presented to the mass spectrometer using a stream of helium, has resulted in a revolution in the use of IRMS analysis in ecological research. The isotopic ratios of analytes present at very low concentrations can now be estimated and the throughput of samples has increased substantially.

There are different types of IRMS systems depending on the type of sample introduction system and/or the method of conversion to light gases. The dual inlet (DI) configuration gives the highest precision in isotope ratio measurements (SD <0.1‰ for ^{13}C and ^{15}N at natural abundance). Such precision is obtained by making repeated comparisons between the sample and a reference gas in each run. The drawbacks are low sample throughput and the amount of manual work required by off-line preparation procedures. The DI technique was not used for the work presented in this thesis.

The CF-IRMS systems were developed in order to obtain faster and more automated instruments. They are all based on on-line conversion of analytes into light gases in a stream of helium prior to detection. With the proper use of reference materials, the precision obtained is only slightly lower (SD ≈ 0.2 ‰) than that for the DI configuration. The precision of CF-IRMS systems is highest for natural abundance work making them suitable for many ecological applications where high precision is required. In the following sections, three types of CF-IRMS systems will be described: Elemental analysis (EA)-IRMS, gas chromatography (GC)-IRMS and liquid chromatography (LC)-IRMS.

Elemental Analysis-IRMS

The interfacing of an elemental analyser with an IRMS was first described in 1983 for nitrogen (Preston and Owens, 1983) and a few years later the first commercial instrument was available (Barrie and Prosser, 1996). The principle is schematically:

Sample introduction → Chemical conversion → GC separation → IRMS

A sample of either a solid (typically 2 mg) or a small volume of non-volatile liquid (typically 2 µL) is placed in a capsule, usually made of tin, and introduced into an elemental analyser (EA) where it is combusted. Thereafter, the combustion products are transported through a reduction furnace (where NO_x is reduced to N_2) and a water trap, followed by a CO_2-trap (in nitrogen analysis only) and a packed GC column (separates N_2 and CO_2) before the sample is carried into the IRMS. Nitrogen and carbon can be analysed in the same run.

Since the chemical conversion takes place *before* separation, bulk isotopic ratios in the sample are obtained. The minimum amount needed for accurate and precise analysis of nitrogen varies depending on instrument manufacturer, but is typically about 50-100 µg N. If the ratio of a specific compound is to be analysed with this technique, then the compound must be isolated from the bulk matrix prior to analysis. An example of such an approach is analysis of ammonium in liquid samples after diffusion into an acid trap (*e.g.* Brooks *et al.* (1989), among others), see later in the thesis.

Gas Chromatography-IRMS

The principle of GC –IRMS is:

Sample introduction → GC separation → Chemical conversion → IRMS

Here the chemical conversion takes place *after* the separation step, and hence the isotopic ratio of individual compounds in the GC effluent can be obtained. This process is also called compound specific isotope analysis (CSIA; Brand *et al.*, 1994).

The hyphenated technique based on the use of a conventional capillary GC in series with combustion and IRMS detection is referred to by several names in the literature; GC – Combustion – IRMS (GC-C-IRMS), isotope ratio monitoring GC-MS (irmGC-MS) and CSIA. It was first described in 1978 by Matthews and Hayes. The use of GC-C-IMRS for CSIA of nitrogen (mainly amino acids) gained popularity about ten years ago (Merritt and Hayes, 1994; Preston and Slater, 1994; Brand *et al.*, 1994). The term GC-C-IRMS will be used in this thesis and it is also the technique used in Papers **I-IV**.

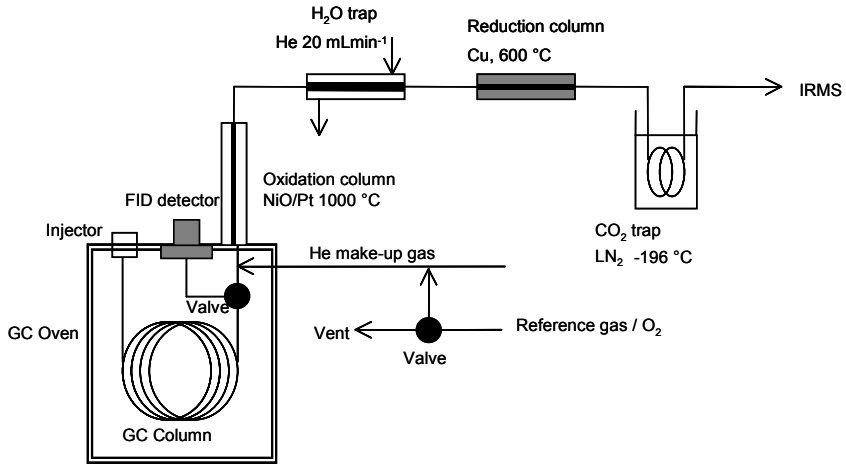

Figure 3. The GC-C-IRMS system used in Papers **I-IV** in this thesis

A small volume (μL) of sample is either injected as a liquid (organic solvent) or as a gas into the GC. After separation in the GC column, it passes through an oxidation column, a Nafion® water trap, a reduction column, and a CO_2-trap before it enters the IRMS (Figure 3, also includes system details). The two last stages before detection, reduction and CO_2 trapping, are used in ^{15}N studies only. The low flow rates used in the GC – C – IRMS system are more compatible with the low pressures in the MS compared to the higher flow rates in EA-IRMS. Thus, a larger part of the gas flow is directed into the MS meaning that a much smaller amount of analyte can be injected without compromising analytical precision. Detection limits vary depending on instrument manufacturer, for nitrogen, values around 0.1 μg N are commonly reported, but values as low as 0.014 μg have been published (SD=0.1-1.0 ‰; Metges *et al.*, 1996). The sensitivity of a GC - C – IRMS system is, however, lower compared to a GC – MS mostly because of the very low abundance of the minor isotope for light elements, which then limits the analysis. In part, the lower sensitivity is also due to the different conversion steps prior to the IRMS, (*e.g.*, oxidation and reduction); these contribute significantly to the band broadening of the analyte, i.e. the pulse of analyte gas along the stream of helium gas becomes more diffuse, due to disturbances in the flow when the tubing diameter in changed. The connectors between the different columns, traps etc. also constitute risks for leaks, which increase the background signal in nitrogen analysis.

The introduction of the GC-C-IRMS technique makes it possible to perform compound-specific isotope analysis of single organic molecules like amino acids (after derivatization, if necessary, to increase their volatility), as has been described in several papers, *e.g.* by Matthews and Hayes (1978).

A packed GC column coupled to an IRMS can be used for analysis of atmospheric gases, for example CO_2, N_2, N_2O and CH_4, and has a wide range of applications in environmental research such as soil respiration and denitrification studies.

Liquid chromatography- IRMS

A few articles have been published describing the coupling of a liquid chromatograph to an IRMS. The very first attempts used a moving belt technique (Caimi and Brenna, 1993), or a thermo-spray/particle beam interface (Teffera *et al.*, 1996), to remove the solvent before sample introduction into the IRMS for ^{13}C analysis. These two techniques are the only means by which the organic solvent can be fully removed, an important aspect for ^{13}C analysis. Recently, the first commercial LC-IRMS instrument was introduced. It is designed for ^{13}C analysis only and is based on chemical oxidation to CO_2, directly in the liquid phase, followed by a gas separator prior to the IRMS (Krummen *et al.*, 2004).

Nitrogen versus Carbon Isotope Analysis

Carbon isotope analysis is, by far, more frequently performed than nitrogen isotope analysis. This is mostly due to the smaller diversity of organic compounds which contain nitrogen. However, analysis of nitrogen isotopes is also, for many reasons, more challenging than analysis of carbon isotopes (Brand *et al.*, 1994; Barrie and Prosser, 1996); (i) In most organic molecules the abundance of nitrogen (< 10 %) is generally less than of carbon (> 60 %). For example, amino acids contain between 2 and 11 carbon atoms, but only 1-4 nitrogen atoms (commonly only one). (ii) Nitrogen is detected as N_2, requiring two nitrogen atoms per gas molecule, while carbon is detected as CO_2, hence requiring only one atom per gas molecule. (iii) The ionization efficiency in the mass spectrometer is lower for N_2, than for CO_2. (iv) The natural abundance of the heavier isotope is higher for carbon compared to nitrogen. (v) Incomplete combustion can lead to interference from CO at m/z 28 (the mass of N_2), and finally (vi) the higher abundance of N_2 in the air constitutes a contamination risk.

For an organic molecule containing 5 wt % N and 60 wt % C, a 50 times higher amount of sample is needed for N than for C to obtain a comparable signal at the minor ion current (Brand et al., 1994). On the other hand, analysis of nitrogen has advantages over analysis of carbon; (i) Nitrogen is not normally incorporated during derivatization of non-volatile compounds prior to gas chromatographic analysis, thus the procedure for correction of isotope dilution, required for ^{13}C analysis, can be omitted and (ii) there is no interference due to column bleeding during the analysis.

Use of Nitrogen Stable Isotopes in Ecological Research

The abundance of ^{15}N shows natural variation in different biospheric pools due to isotopic fractionation in different chemical and physical processes. Typically the deviation of biological samples from the ratio in atmospheric N has a narrow range of between –0.0040 to +0.0060 atom % (≈ -10‰ to +20 ‰) (Högberg, 1997). The use of stable isotope analysis constitutes a unique possibility to study

these processes in different ecosystems, as well as in other fields such as physiology and forensic science. Reviews by Handley and Raven (1992) and Högberg (1997) give detailed information about different processes leading to nitrogen isotopic fractionation in soil-plant systems. As an example, Simpson *et al.* (1999) demonstrated that compound-specific nitrogen analysis of amino acids could be used as an indicator of former land use.

^{15}N tracer studies, where ^{15}N-labelled compounds and their reaction products are monitored, can also be used to study processes and mechanisms of nitrogen cycling in ecosystems. For example, pool dilution experiments have been used to study turnover rates of inorganic nitrogen pools in different soils (*e.g*. Davidson *et al.*, 1991). In other tracer experiments, the use of both ^{13}C- and ^{15}N- labelled glycine in field trials has been used to show that glycine can be taken up by different plants in its intact form (Näsholm *et al.*, 1998) and that it is used directly by micro organisms in the soil (Barraclough, 1997).

Methodology for ^{15}N Analysis in Soil

Correctly designed field or laboratory experiments are important when studying nitrogen isotopes in soils. However, besides this, there is also a need for precise and accurate analytical methods. Formerly off-line preparation techniques, where the species of interest are separated from a matrix, combined with on- or off-line oxidation and IRMS detection, were the dominant procedures for nitrogen isotope analysis of individual compounds. During recent decades, the use of the GC-C-IRMS technique for compound-specific isotope analysis has become more prevalent. In Figure 4, generalised sample preparation schedules for off- and on-line (GC-C-IRMS) separation for nitrogenous compound analysis of a soil sample are presented.

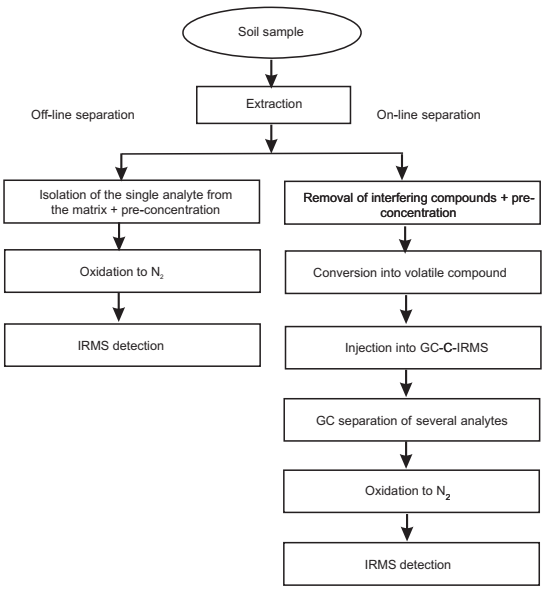

Figure 4. Outline sample preparation schemes for nitrogen CSIA of soils.

When the soil is sampled, a suitable extraction technique must be chosen for the compound(s) under consideration. Different extraction techniques were not evaluated in this thesis, but are briefly discussed. Individual nitrogenous compounds in forest soil are usually present in very low concentrations, this fact in combination with the relatively lower sensitivity in IRMS analysis (compared to other detection techniques like Flame Ionization Detection (FID) and organic MS) and a complex sample matrix makes sample preparation complicated. Sample preparation and the inherent risks of isotopic fractionation at the different stages is often the greatest limitation on the analytical performance of IRMS (Meier-Augenstein, 2004). In the papers on which this thesis is based, some of these steps were studied, including pre-concentration (Papers **II-IV**) and removal of interfering substances during sample preparation (Paper **IV**).

Objectives

The specific objectives within this thesis are:

- To develop analytical methods for nitrogen CSIA for ammonium in aqueous solutions (with the extension to soil extracts), using GC-C-IRMS (Papers **I, II** and **III**).
- To develop a method for nitrogen CSIA for the free glycine pool in soils, using GC-C-IRMS (Paper **IV**).
- To develop procedures using new materials for sample preparation and pre-concentration prior to GC-C-IRMS analysis, and to evaluate their effects on the measured isotopic ratio (Papers (**II** and **III**).

Extraction of Nitrogen Species from Soil Samples

Traditional extraction and sample preparation techniques used for quantitative analysis of nitrogenous compounds in soils (see The Soil Science Society of America book series *"Methods of Soil Analysis"* (Bartels, 1996) for accounts of the most common methods) have also been applied to nitrogen isotope analysis of compounds. Individual nitrogenous compounds that are not bound into polymeric structures such as peptides are usually extracted from soils with an aqueous solution followed by filtration of the soil solution slurry. The procedures used for extraction ideally should not alter the composition of the sample, which for isotope analysis also includes preservation of the isotopic ratios of species of interest. However, there is no guarantee that the $\delta^{15}N$ in the pool extracted does exactly reflect the $\delta^{15}N$ pool in the soil. Lindau and Spalding (1984) found a +6.2 ‰ shift in the $\delta^{15}N$ of nitrate when increasing the soil-to-extractant ratio from 1:1 to 1:10.

Although the studies covered in this thesis have not been focused on extraction methods, the procedures used for extraction of ammonium and glycine from soils are briefly discussed below.

Liquid Extraction

Ammonium in soil consists of fixed and exchangeable fractions. The latter is the more active pool in soil – plant interactions and includes ammonium that is free in the soil solution or bound to negatively charged soil particles by ionic attraction. Exchangeable ammonium is commonly extracted using a KCl solution. A concentration of 2 M and a soil-to-extractant ratio of 1:10 has been shown to quantitatively remove exchangeable ammonium from different soil types (Bremner and Keeney, 1966) by a cation exchange mechanism.

As part of the method development described in Papers **I** and **II**, simulated soil extracts were prepared using a 4 M KCl solution because this higher salt concentration was shown to increase the sensitivity of the methods presented. However, it has been reported that even highest analytical quality KCl salt may be contaminated by NH_4^+ at concentrations sufficient for significant alteration of ammonium nitrogen isotopic composition in liquid extracts. The NH_4^+ concentration is not consistent between different lot numbers from the same manufacturer and therefore should be checked before the salt is used for low concentration work.

Free amino acids (FAA) in soils are here defined as AA that appear freely in soil solution or that are loosely bound to soil organic or particulate matter and are not bound in peptides or other organic compounds. There is no generally accepted extraction technique for FAA. Extraction with 20 % ethanol (Gilbert and Altman, 1966), as well as water (Paul and Schmidt, 1960), has been reported to be efficient for acidic and neutral FAA. For basic FAA, both $Ba(OH)_2$ and NH_4OAc were more efficient as extraction solutions (Paul and Schmidt, 1960). For some FAA, the use of $Ba(OH)_2$ gave a recovery in excess of 100 % for 'spiked' soils, indicating possible hydrolysis of peptides during sample preparation. Both carbon tetrachloride (Sowden and Ivarson, 1966) and 2 M KCl (Jones *et al.*, 2002) are suspected of decomposing microbial cells or other cellular materials during extraction. Ivarson and Sowden (1969) suggested that water would probably give the best estimate of FAA under natural conditions, due to the risk of contribution from protein-bound AA when using other extractants. Water was also the extractant used in Paper **IV**.

Centrifugation

Centrifugation of the soil can give time-specific estimates of the concentrations of different chemical species in the soil solution. Monreal and McGill (1985) found that the centrifugation procedure did not destroy microbial cells, thus making it useful for extraction of FAA in the soil solution. Compared to zero-tension lysimetry, Giesler *et al.* (1996) concluded that centrifugation might better reflect the plant-available concentrations in the soil solution. For the method development work for free glycine analysis in Paper **IV,** centrifugation was also used as well as water extraction.

Nitrogen Compound Specific Isotope Analysis

Ammonium and glycine, the compounds that are dealt with in this thesis, have differing chemical properties. Consequently, different approaches were used for nitrogen isotope analysis of the two species in liquid matrices and therefore they are treated in separate chapters in the thesis.

In the literature, the term CSIA is used for isotope analysis of individual compounds after separation by GC (Brand *et al.*, 1994). In this chapter the term is used also for off-line methods for ammonium since they are, in fact, compound specific.

Ammonium

Ammonium is an interesting species for several reasons. Firstly, it is one of the main nitrogen species taken up by plants in both marine and terrestrial ecosystems. Secondly, in traditional soil analysis, most nitrogen pools in organic material are converted to ammonium by Kjeldahl digestion (H_2SO_4) prior to purification (distillation) and quantification (Hauk *et al.*, 1994). A method for isotope analysis of ammonium is therefore applicable to several soil nitrogen pools.

For quantification of total nitrogen, soil is directly digested, while hydrolyzable N fractions being released by treatment with hot HCl (usually 6 M) and thereafter separated into total hydrolyzable N (by digestion + distillation), amino acid N (by ninhydrin treatment + digestion + distillation) and the ammonium and ammonium + amino sugar (by distillation at different pH values). The concentration of other N fractions can be inferred by subtraction. Nitrate can be reduced to ammonium by the use of Devarda´s alloy (50 % Cu, 45 % Al, 5 % Zn), which has also been used in ^{15}N studies (O´Deen, 1980).

Due to the high sensitivity of different wet chemical methods, these are commonly used for quantification of ammonium in low-concentration samples. The classical indophenol blue method uses a reaction between ammonium and phenol, in the presence of hypochlorite, followed by spectrophotometric detection. The reaction and detection can either be performed manually or using an automated Flow Injection System (FIA). Detection limits around 10 μg L^{-1} are reported for routine laboratories and only a few mL of sample are required for FIA analysis.

^{15}N Analysis of Ammonium with Off-line Separation

Various off-line sample preparation techniques have been used for compound-specific isotope analysis of ammonium. As shown in Figure 4, the following steps are generally included: (i) Off-line separation from the liquid matrix and pre-concentration, (ii) Conversion (oxidation) to N_2, and (iii) IRMS detection.

Separation from the liquid matrix is either based on ion exchange (*e.g.* Lehmann *et al.,* 2001), derivatization (Johnston *et al.*, 2003) or phase-transfer. Phase transfer is performed by conversion of ammonium into ammonia followed by steam distillation (*e.g.* Buresh, 1982) or by diffusion (*e.g.* Brooks *et al.,* 1989) into an acid trap. The steam distillation and diffusion procedures are described in detail by Mulvaney (1993). It is not possible to use the ion exchange approach with 2 M KCl soil extracts due to their high concentration of potassium ions, but all the other approaches have been successfully used. Since the diffusion technique is the most common technique for separation of ammonium from the matrix prior to isotope analysis nowadays, it is described more in detail below.

There are three principal steps in the *diffusion* technique: (i) the pH of the aqueous sample is increased in order to convert ammonium to ammonia, (ii) ammonia is diffused from the sample into an acidified trap, *e.g.* an acidified filter paper hanging above the surface (e.g. Brooks et al., 1989) or an acidified filter paper enclosed in PTFE film (e.g. Sörensen and Jensen, 1991) in a closed vessel and finally (iii) after diffusion is complete, the trap is analysed, usually as a solid sample using EA-IRMS. A schematic picture of the diffusion is shown in Figure 5.

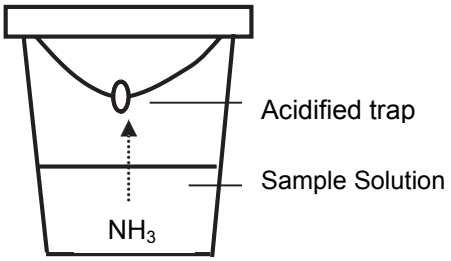

Figure 5. Schematic picture of the diffusion technique used for isolation of ammonium (after conversion to ammonia) from a liquid matrix prior to nitrogen isotope analysis, using an acidified filter paper hanging above the surface as trap.

Incomplete recovery in the diffusion process has been reported, a problem more commonly reported for solutions with low nitrogen content (O´Deen, 1980) and also for large diffusion volumes (O´Deen, 1979; Saghir, 1993), however the relationship between recovery and measured isotopic ratio has not been clarified. The minimum concentration for soil extracts analysed using the diffusion technique was about 0.5 mg N L^{-1} (Kelly *et al.*, 1991; Stark and Hart, 1996), but for seawater samples, Holmes *et al* (1998) have successfully diffused up to 4 litres of samples at a concentration of only 0.007 mg N L^{-1}.

The precision reported for the different separation techniques is generally high, with SD <0.3 ‰ being reported for the ion exchange method and 0.23 ‰ for the derivatization procedure (both at natural abundance concentrations). The diffusion and distillation techniques are less commonly used for natural abundance work, but SDs around 2-3 ‰ have been reported for diffusion (Brook *et al.*, 1989) and the distillation was reported to give a precision which was slightly worse when comparing the two techniques (Adamsen and Reeder, 1983).

Irrespective of the sample preparation technique used, it is the IRMS instrumentation *per se* which determines the minimum amount of nitrogen required for analysis, and with the EA-IRMS approximately 50-100 µg of N is needed for high precision estimates of the isotopic ratio. The concentration of ammonium varies among KCl soil extracts, but an average value of 0.46 mg N L^{-1} has been reported for different soil types in Ireland (Jones*, et al.*, 2002). They also reported values below 0.3 mg N L^{-1} for some sites. Hence, for the off-line methods described above, ≈ 150-300 mL of extract are needed, a sample volume which is too large for most distillation systems and also problematic for diffusion.

Improvements to relevant instrumentation are, therefore, of interest. Fry *et al.* (1996) presented a cryoflow system to be used with an EA-IRMS system where the gases produced after combustion were separated by the use of different traps and volatilized into the IRMS one by one at a much lower flow rate. Fry *et al.* (1996) successfully worked with samples containing < 0.03 mg N L^{-1}, reporting good reproducibility for routine analysis of samples containing as little as 50 nanomoles of C, S and N (0.7 µg N). They also performed analysis of $\delta^{15}N$ in ammonium after diffusion from a 50 mL liquid sample containing only 1.4 µg N. However, no such commercial system is currently available.

Due to the much smaller amount of nitrogen needed for isotope analysis using GC-C-IRMS, and also due to the on-line separation provided, the possibilities to use this technique for ammonium were evaluated (Papers **I-III**).

*^{15}N Analysis of Ammonium by GC-C-IRMS (Papers **I-III**)*

As mentioned previously, wet chemical methods are the most sensitive for quantification of ammonium. Hence, gas chromatographic analysis of ammonium (after derivatization) is only used for ^{15}N isotope analysis. In the GC methods described here for measuring ^{15}N enrichment of ammonium, conventional organic mass spectrometry has been used for detection. Bengtsson and Bergwall (2000) measured ^{15}N enrichment in ammonium from soil after conversion to pentafluorobenzamide, a derivatization technique described by Fujihara *et al.* (1986). The derivatization technique worked even for samples of high ionic strength. Fluoro-compounds have been shown to irreversibly damage the oxidation column in GC-C-IRMS (Meier-Augenstein, 1997), however, by observing the performance of the oxidation column and replacing it before the end of its lifetime, fluoro-compounds may be analysed. Preston *et al.* (1996) formed indophenol by reacting ammonium with phenol. Indophenol was then extracted out of the solution (sea water) with a C_{18} extraction disc, dried and further derivatized to bis(trimethylsilyl)-indophenol and subjected to ^{15}N analysis. The

derivatization introduced a large amount of carbon (18 carbon atoms per molecule), which constitutes a risk for incomplete combustion in a GC-C-IRMS system. Also, the procedure has been evaluated for natural abundance with IRMS detection and was found to perform poorly (Johnston *et al.*, 2003).

In Papers **I-III** in this thesis, the same phase-transfer principle as used in the diffusion and distillation procedures described above, namely conversion of ammonium to ammonia, was used in combination with headspace analysis of ammonia for nitrogen isotope analysis. The headspace technique was used as a direct technique (Paper **I**) and in combination with different materials for pre-concentration (Papers **II** and **III**). Ammonia gas has previously been successfully analysed for ^{15}N by GC-C-IRMS with a detection limit around 0.16 μg of N (Lauf and Gebauer, 1998).

Equilibrium Headspace Sampling

In equilibrium (also called static) headspace (HS) analysis, the headspace gas volume is that above a liquid sample in a sealed container (Kolb and Ettre, 1997). The analytes are allowed to equilibrate between the liquid and the head space gas, an equilibrium which can be described by the dimensionless Henry´s law constant according to:

$$K_H = \frac{\gamma_S \cdot C_S}{C_G} \tag{7}$$

where C_S and C_G (both in moles L^{-1}) are the concentrations of a compound in the sample (usually liquid, but can also be a solid) and gas phase, respectively, and γ_s is the activity coefficient of the analyte in the condensed phase. After equilibrium is reached, an aliquot (typically 25-250 μL with split-less injection) of the headspace gas is injected into a GC system and the concentration in the sample can be quantified from the equation above.

Equilibrium headspace analysis with GC-C-IRMS has been used for ^{13}C (Slater *et al.*, 1999; Ahad *et al.*, 2000) and ^{2}H (Ward *et al.*, 2000) in organic contaminants and ^{13}C in dissolved organic carbon (Miyajima *et al.*, 1995), but was firstly reported for ^{15}N in Paper **I** in this thesis. An advantage of using headspace analysis with GC-C-IRMS is that a gas sample without extraneous C is injected into the GC, hence saving the oxidation column from the risk of unnecessary carbon exposure, which otherwise rapidly reduces the oxidation potential of the NiO packing material. In addition, large amounts of carbon also plug the liquid nitrogen CO_2-trap used in nitrogen isotope analysis.

The sensitivity of headspace analysis is inversely proportional to the K_H value, consequently isotope analysis or general quantification of volatile organic compounds is the dominant application for headspace analysis. However, for readily soluble compounds with high K_H values, (*e.g.* ammonia, see Hales and Drewes (1979) and Dasgupta and Dong (1986)), the sensitivity can be improved by optimisation of factors such as gas:liquid ratio (β), equilibration temperature (T) and salt concentration (affects γ_s) (Kolb and Ettre, 1997). In Paper **I**, the concentration of ammonia in the gas phase was maximised for equilibrium headspace analysis of ammonium in simulated soil extracts, the ammonium in

solution being completely converted to ammonia by increasing solution pH. It was shown that a high salt concentration in combination with a high temperature and a low β should preferably be used, which is in agreement with the conditions favouring headspace analysis of aliphatic amines in aqueous samples (Maris *et al.*, 1999).

Thermodynamic isotopic fractionation of analytes can occur between the dissolved fraction and the fraction present in the gas phase. However, a thermodynamic isotopic fractionation is constant under given laboratory conditions (temperature, etc.) and can be corrected for by using standards with known isotopic composition. In Paper **I**, the liquid: gas equilibrium isotopic fractionation factor ($\alpha_{eq}=R_{liquid}/R_{gas}$, where $R=^{15}N/^{14}N$) for ammonia was determined to 1.0054±0.0007, which is in agreement with previously determined values (Thode, 1945; Kirshenbaum *et al.*, 1947), but with a higher precision.

Ammonia shows a larger liquid:gas isotopic fractionation than other compounds studied such as inorganic C, for example, Miyajima*, et al.* (1995) reported a fractionation factor of 1.0011 for $\delta^{13}C$ in dissolved inorganic carbon in water. Generally, organic molecules show an isotopic fractionation factor below 1 in the equilibrium between liquid and gas phases, showing that fraction in the gas phase is enriched, a phenomenon, called the "inverse isotope effect". Equilibrium fractionation factors (α_{eq}) of 0.9991 and 0.9987 have been reported for carbon in trichloroethylene (TCE) and dichloromethane, respectively (Huang *et al.*, 1999). However, Slater *et al.* (1999) did not see a statistically significant enrichment of ^{13}C in the gaseous phase when performing headspace analysis of toluene and TCE.

When performing nitrogen isotope analysis, there is always a risk of contamination from atmospheric nitrogen. Although a gas-tight syringe was used for injection of the gaseous ammonia and the headspace in the sample tubes was purged with helium before conversion of ammonium to ammonia (Paper **I**), small traces of nitrogen gas were always co-injected with the ammonia. A complete separation between ammonia and nitrogen in the gas chromatographic step was therefore important and was achieved using a PoraPLOT Amines capillary column. This column is specially designed for separation of ammonia and volatile amines and has also been used for headspace analysis of C_1-C_4 aliphatic amines (Maris*, et al.*, 1999). The method in Paper **I** has obvious potential to be extended to include ^{15}N analysis of both ammonia and amines in the same chromatographic run.

The precision obtained for ammonia at natural abundance (Paper **I**; SD ≈ 0.6 ‰ for experimental replicates) with only 0.9 μg of N injected (in 250 μL headspace gas), was high and comparable to the precision obtained using off-line preparation techniques. The method was also used for more enriched ammonium standards with promising results. However, the liquid concentration of ammonium required to give a stable and precise value in the isotopic ratio estimate was 420 mg L^{-1}, (30 mM) which is too high to be used for forest soil sample extracts, but may be of use for agricultural soils and also for Kjeldahl extracts and in studies of ammonia volatilization from manure (Sommer and Olesen, 2000).
A theoretical discussion of how to increase further the sensitivity of equilibrium headspace analysis was included in Paper **I,** showing that the "full evaporation

technique" (FET; the whole headspace volume injected into the GC, preferably with a high β value) can be used for samples with an ammonia concentration in the low mg L^{-1} range. This is in the same concentration range as that required for the diffusion technique, but a smaller sample volume is needed for FET. This technique would require a large volume injector and a cold trap to reduce the band broadening which occurs during injection. The injection of large amounts may also lead to blocking of the cold trap by condensation of the co-injected water vapour. However, with an aqueous sample at 80 °C, an injection time of several minutes can be used before a cold trap with 0.53 mm i.d. becomes plugged (Kolb and Ettre, 1997).

Dynamic headspace analysis can also be used to increase the sensitivity relative to that of the static headspace technique. It involves purging of the sample, cryogenic trapping of analyte(s) from the purge gas flow, and subsequent release onto the GC column. Dynamic headspace analysis has, for example, been used for carbon isotope analysis of dissolved chlorinated ethenes (Morrill *et al.*, 2004). For these analytes, the isotopic fractionation between liquid and gas phase was smaller than the measurement uncertainty and accordingly the measured ratio was independent of purging efficiency. For ammonia, on the other hand, an isotopic fractionation is expected and a quantitative recovery during purging is more important. However, for compounds with a high K_H value, the rate at which they are purged out of solution is slow. A purge time of 3 hours was needed to quantitatively purge ammonia (0.2-2 mg L^{-1} range tested) out of a solution volume of 5 mL at 10 mL min^{-1} for a sample kept at 90 °C (Paper **III**). Besides the inconvenience of only running one sample every 3 hours, the cryo-trap will quickly become plugged by condensation of the associated water vapour. Other ways to increase the sensitivity in headspace analysis were evaluated in Papers **II** and **III**, and these are described in the following chapters of this thesis.

Solid Phase Microextraction

Solid Phase Micro Extraction (SPME) is an extraction technique which was first described in 1990 for volatile organic compounds in water (Arthur and Pawliszyn, 1990) and commercially produced syringe and fibre assemblies became available in 1992. It is based on absorption or adsorption of one or several analytes from a gaseous or liquid phase onto a coated fused silica fibre, followed by thermal desorption in a GC injection port. Complete extraction from the sample matrix is not necessary (Arthur and Pawliszyn, 1990). A schematic picture is shown in Figure 6.

Figure 6. Exposure of an SPME fibre to the headspace above a liquid sample. The fibre is thereafter thermally desorbed in the GC injection port. Republished with permission from Supelco, a subsidiary of the Sigma-Aldrich Corporation.

The amount absorbed onto the fibre is dependent on the concentration in the sample. When absorbing from the headspace above a liquid, the fibre-sample partition coefficient, K_{fs}, is a combination of the Henry´s law constant, K_H, and the and the fibre-gas partitioning coefficient K_{fg};

$$K_{fs} = \frac{K_{fg}}{K_H} \tag{8}$$

The concentrating effect of properly selected fibres means that analytical sensitivity is higher than that of direct injection of headspace gases. Like the equilibrium headspace technique, SPME is suitable for GC-C-IRMS analysis since the analytes are extracted, concentrated and injected without the use of solvent. Apart from the isotopic fractionation which can occur for analytes in the equilibrium between liquid and gas phases, the SPME absorption step may introduce further fractionation in the phase partitioning between the gas and fibre for the compounds studied.

The coatings of commercially-produced SPME fibres available for selective trapping of analytes are non- or slightly-polar, suitable for use with compounds with similar properties, preferably dissolved in polar solvents. Consequently, the first work using SPME in combination with GC-C-IRMS dealt with measurement of ^{13}C at natural isotopic abundance levels in organic acids and hydrocarbons from aqueous solutions (Dias and Freeman, 1997). More recent publications are sparse, but do occur, for example chlorinated compounds and flavours have been analysed for ^{13}C (Dayan *et al.*, 1999; Hunkeler and Aravena, 2000; Goupry *et al.*, 2000), alcohols analysed for ^{13}C and ^{18}O (Aguilar-Cisneros *et al.*, 2002) and *tert*-butyl ether analysed for ^{2}H (Gray *et al.*, 2002).

In 1998, Górecki *et al.* extended the field of applications for SPME by using a fibre coated with a perfluorinated membrane (Nafion®). It was superior to the commercial coatings for analysis of polar compounds (like alcohols and water) in non-polar matrices, but also suitable for the most polar compounds (like methanol) in water. In the absence of commercially-available polar coatings suitable for

nitrogen isotope analysis of ammonia SPME assemblies were constructed, using metal capillary tubing and optical fibres which were coated with Nafion®, in-house (Paper **II**). Paper **II** deals with both the construction and evaluation of the SPME assemblies produced in-house. The hypothesis was that Nafion® would act as an acid trap, where the sulphonate groups in its matrix and the ammonia molecule form an ammonium salt, a reaction which is reversed in the GC injection port by increasing the temperature (thermal desorption).

The fibres were initially evaluated for ammonia gas and an isotopic fractionation factor about 1.01 was found between the ammonia absorbed in the fibre and ammonia in the gas phase within a gas concentration range of 21-210 µM. A gas phase concentration of 21 µM resulted in desorption of 0.5 µg of N, which was considered to be the limit of detection for N isotope analysis. When absorbing from a liquid sample, an initial concentration of 140 mg N L^{-1} (10 mM) was needed in order to detect 0.5 µg, which is 3 times more sensitive than the equilibrium headspace technique in Paper **I**. However, the precision obtained was worse (SD= 1.8-4.0 ‰ for 140-700 mg N L^{-1}). Unfortunately, the fractionation factor between liquid and fibre could not be determined, since no isotopically calibrated ammonia was available, but a value lower than the 1.01 reported between fibre and gas is expected, since ammonia in equilibrium between a liquid and a gaseous phase show an isotopic depletion in the gaseous phase (reported in Paper **I**).

However, the fractionation factor obtained for ammonia between fibre and gas is large compared to the fractionation factors below 1.001 reported for ^{13}C when applying SPME and GC-C-IRMS to toluene, methylcyclohexane, hexanol, (Dias and Freeman, 1997) and chlorinated methanes, ethanes and ethenes (Hunkeler and Aravena, 2000).

In Paper **II**, the Nafion® fibre was also evaluated for absorption from one single droplet. Liu and Dasgupta (1995) found that an acidified liquid droplet was a good trap for the sampling of ammonia from an air sample and it could, therefore, potentially be used as the acid trap when diffusing ammonia from a liquid sample. The intention in Paper **II** was to use the SPME procedure as the last step after diffusion of ammonia into an acid droplet. This would then reduce the amount of ammonium needed compared to direct SPME absorption from a liquid sample. The isotopic ratio for five replicates, containing only ≈ 5.6-7 µg N at natural isotopic abundance, was measured with high precision (SD = 0.8 ‰). These pilot experiments were promising and led to further investigations.

Besides the experiments presented in Paper **II**, single droplets of standard ammonia solutions, containing different amounts of N (7.5-50 µg) and of differing isotopic compositions (57-4800 ‰) were used for absorption, according to the procedure described in Paper **II** ("Absorption from a droplet"). The use of enriched solutions led to the finding that there was a memory effect in the fibres, which affected the measured isotopic value for the next sample. This phenomenon was not found when working at natural abundance in Paper **II**. An explanation could be that the Nafion® matrix changes in structure when its water content is reduced below ≈ 20 % (v/v), leading to isolated ionic zones in the interior of the fibre material (Gebel, 2000). Most probably this occurred at the high temperature

(175 °C) used in the thermal desorption step, and ammonia was trapped in these isolated zones. Neither an increase nor a decrease of the desorption temperature increased the yield of ammonia in the desorption process.

No trace of the enriched ammonia remained following acid washing of the Nafion® fibres by boiling in 1 M H_2SO_4 for one hour, this was confirmed by an accurately measured isotopic value from the subsequent absorption. Since most of the absorbed ammonia was trapped within the matrix and not desorbed by heat it was found that at least 30 µg N was required in the droplet. The amount needed was then only 1-2 times lower than the amount required for isotope analysis of a solid sample using EA-IRMS (*e.g.* a filter paper trap after an ammonia diffusion procedure). Thus, bearing in mind the labour intensity of the SPME technique, the advantages were found to be too small for use in routine analysis.

Purge and Trap

The purge and trap (P & T) technique can be used to extract and concentrate analytes from a sample prior to analysis. The liquid sample is purged with an inert gas and the gas passed through an adsorbent, which ideally quantitatively traps the analyte(s). After trapping, the analytes are thermally desorbed and passed through a GC separation column before detection. Depending on the kinetics in the desorption step, a cold trap may be needed. The sensitivity when using P & T is directly proportional to the efficiency in the purging step and the time needed for quantitative purging is dependent on several factors, such as sample volume, K_H, and purge flow rate. Off-line P & T makes it possible to process several sample tubes simultaneously, which increases sample throughput compared to dynamic headspace with a cold-trap, which has been described previously in this thesis. P & T has been used in carbon isotope analysis of different volatile organic compounds such as chloroform, benzene, toluene, *tert*-butyl ether, chlorinated ethylene and tetrachloroethylene (*e.g.* Beneteau *et al.*, 1999 and Zwank *et al.*, 2003).

In Paper **III**, a sulphonated styrene/divinylbenzene copolymer absorbent for P & T of ammonia was developed, and evaluated for its use with nitrogen isotope analysis. Again, ammonium was completely converted to ammonia in an aqueous matrix followed by purging of the sample and trapping onto the absorbent. The mechanism of trapping is the same as with the Nafion® coated SPME fibre described in Paper **II**, i.e. formation of an ammonium salt between the sulphonate groups in the absorbent matrix and the ammonia molecules.

The measured isotopic value for ammonia was shown to vary largely. For a solution containing 10 µg N at 426 ‰, purged at 5 mL min^{-1}, the measured isotopic ratio after desorption ranged between 278 and 410 ‰ (n=3). The amount thermally desorbed ranged between 3.6 and 6.0 µg N for the tubes. After thermal desorption, another 0.1-0.6 µg N was eluted with acid. The amount not recovered (3.7-5.8 µg N) was probably not trapped, but passed through the tubes during the trapping procedure, a phenomenon which could partly be described by the residence time in the adsorption tubes being too short for all ammonia to diffuse onto the adsorbent coating on the tube wall. However, an alternative explanation

for the poor recovery is that the number of adsorption sites was insufficient for the number of ammonia molecules present in the sample.

The most important factor determining the measured isotopic ratio was the efficiency of the desorption step. A temperature of 300 °C was needed for the desorption process and efficiency was not increased by increasing the time from 20 to 30 minutes. There was a linear relationship between the proportion of ammonia which was not thermally desorbed (undesorbed/total amount trapped (= x)) and the measured isotopic value ($y = -1107 \cdot x + 419$, $R^2 = 0.94$). A quantitative desorption was then estimated to give a value of 419 ‰, which is very close to the true isotopic value. Zwank *et al.*, (2003) report a similar phenomenon with non-quantitative desorption when studying ^{13}C in different organic substances. However, the kinetics for their system were much faster and increasing the desorption time from 30 to 60 seconds, eliminated this effect.

The very large influence of the undesorbed ammonia fraction on the measured isotopic ratio makes this method difficult to use. Further investigations are needed in order to diminish the memory effect. Nevertheless, since the sensitivity is directly proportional to the efficiency in the purging step, the P & T technique is an attractive technique. For different organic groundwater contaminants, it was shown to lower the detection limits by more than 4 orders of magnitude compared to liquid on column injection (Zwank *et al.*, 2003). In principle, the P & T technique has the potential to be used for samples containing below 1.0 µg N (the detection limit when analysing ammonia on the instrument used), which is lower that any method available today.

Summary of the Different Methods for Ammonium

None of the three different techniques described in Paper **I-III** have previously been used for determination of ^{15}N in ammonium. However, all these techniques have been successfully used for determination of $\delta^{13}C$ in organic compounds in aqueous matrices. In principle, there are three reasons for the increased difficulty when analysing ammonium: (i) Larger equilibrium isotopic fractionation for the ammonia molecule between liquid and gas phases and larger kinetic fractionation during evaporation than for organic compounds. (ii) Ammonia has a higher K_H than organic compounds, which is disadvantageous for all three techniques discussed. At 25 °C, ammonia shows a K_H of 1300 compared to 3.7 for toluene. (iii) Method development is more straightforward for organic compounds for which commercial materials are available for use with the SPME and P & T techniques since the different procedures have already been optimised. At present there are no commercially available materials suitable for absorption/trapping of ammonia with the SPME and P & T techniques.

Glycine (Paper IV)

Glycine has often been used as a model amino acid in different N cycling studies such as plant uptake (Näsholm*, et al.*, 1998) and also in pool dilution experiments (Barraclough, 1997). Nitrogen isotope analysis of the protein-bound amino acids in soil released after acid hydrolysis has been performed with GC-C-IRMS (Ostle *et al.*, 1999; Simpson*, et al.*, 1999), but Paper **IV** of this thesis instead focuses on the free glycine pool, which is a potential nitrogen source for plants (*e.g.* Nordin *et al.*, 2001; Näsholm*, et al.*, 1998).

Purification and Pre-concentration

Prior to derivatization of a sample for amino acid analysis, the sample generally requires some kind of purification to remove potentially interfering species such as proteins. In addition, pre-concentration may also be necessary, as in the case of $\delta^{15}N$ analysis of free glycine in a soil extract (Paper **IV**).

A cation exchanger is commonly used for isolation of amino acids from a liquid sample, *e.g.*, a protein hydrolysate (Odham and Bengtsson, 1987). After acidification of the sample, neutral and anionic species are passed through a column packed with the cation exchanger, while the amino acids and other cationic species are retained and later eluted. Aqueous ammonia is commonly used as the eluent, and desorbs the amino acids by a combination of cation exchange and increasing pH, which makes the amino acids neutral or anionic. Nowadays, the commercial solid phase extraction (SPE) cartridges are widely used, having the advantage of being user friendly with clearly written instructions. Another approach is to add an ion exchange resin to the sample which is then shaken, filtered, and finally the AA eluted.

Different cationic exchangers have been reported to give variable recovery success for amino acids (Odham and Bengtsson, 1987) and should be evaluated before use. Recovery can also be affected by other species in the solution. Different soil types can have widely differing properties and a procedure used for one soil type may not meet the needs for another. The ion exchange procedure should also be checked for isotope effects. Hofmann *et al.* (1995) observed a –2‰ shift in the $\delta^{15}N$ value for different amino acids in standard solutions when using an Amberlite cation exchanger. This shift was not found for glycine using a commercial cation exchange Solid Phase Extraction (SPE) cartridge (packing material of unknown source) in Paper **IV**.

For soil hydrolysates, with high concentrations of Fe and Al, Amelung and Zhang (2001) obtained a low recovery of amino acids using an ion exchange procedure. They found that Fe and Al were tightly bound to the ion exchanger and made elution of the amino acids ineffective. By pumping an oxalate solution through the ion exchanger before elution, the metal ions were eluted by complexation and the recoveries of different AA during the subsequent alkaline elution were not significantly different from 100 %.

For the soil water extract used in Paper **IV**, the recovery of spiked glycine was approximately 30 % when using a commercial cation exchange SPE cartridge,

while the recovery was not significantly different from 100% for glycine dissolved in deionised water. The high concentration of humic substances (HS) in the soil water extract was suspected to reduce the recovery. HS have a very complex chemical nature with large hydrophobic regions, as well as a number of functional groups, and there could be several mechanisms leading to the reduced recovery for AA. AA are known to adsorb to cation exchange resins by ion attraction as well as molecular adsorption (Odham and Bengtsson, 1987) and it is probable that the humic substances disturbed both mechanisms, with positively charged amino groups competing for the ionic sites and the more hydrophobic parts being attached through molecular adsorption.

It has been shown that humic acids are both physically and chemically bonded to aminopropyl silica (Koopal *et al.*, 1998) and by using an SPE cartridge packed with an aminopropyl weak anion exchanger prior to the cation exchanger, the humic content was reduced and glycine recovery was not significantly different from 100 %.

Derivatization

Amino acids are non-volatile and derivatization is therefore required prior to gas chromatographic separation. Some derivatization techniques used in GC analysis with other detectors (Blau and Halket, 1993) are directly compatible with the GC-C-IRMS technique, whereas others are not.

Both the carboxy- and amino-groups of the amino acids need to be derivatized and there several different derivatization techniques to chose from; silylation, esterification followed by fluoroacetylation, acetylation or pivaloylation and reaction with a chloroformate, usually ethylchloroformate (ECF) (used in Paper **IV**). As concluded by Hofmann *et al.* (2003) after a comparison and evaluation of all the above mentioned techniques with the exception of the reaction with ECF, none of the methods is universally suitable for all proteinogenic amino acids and the choice of method depends on the application.

Derivatization techniques introducing a large number of carbon atoms such as trimethylsilylation (TMS) and, especially *tert*- butyldimethylsilylation (*t*BDMS; giving a C:N ratio of 12 for glycine), can lead to formation of CO if the combustion process is incomplete, resulting in interference with nitrogen (at m/z 28) in the mass spectrometer. Furthermore, the large amount of CO_2 formed during combustion will quickly deplete the oxidation column, and plug the cold trap. There is also a risk of overloading the separation column. TMS has been reported to give several derivatization products for some amino acids (Hofmann*, et al.*, 1995), leading to incorrect isotopic estimates. The silylated derivatives have also been reported to be stable only for a short period of time (Hofmann*, et al.*, 1995), which can be especially critical in isotope analysis.

Esterification followed by fluoroacetylation is a popular derivatization technique since it enhances detection with organic MS because of the highly characteristic patterns in the mass spectra. It has successfully been used for isotope analysis of both carbon (Silfer *et al.*, 1991) and nitrogen (Macko *et al.*, 1997) in amino acid enantiomers. However, for GC-C-IRMS, fluoro-compounds are known to

irreversibly damage the oxidation column by formation of very stable metal fluorides (Meier-Augenstein, 1997). Instead, esterification can be followed by acetylation and pivaloylation, producing derivatives which are totally compatible with GC-C-IRMS analysis (Metges*, et al.*, 1996; Metges and Petzke, 1997). While acetylation is the most common technique, pivaloylation was shown to give derivatives with better chromatographic separation on apolar stationary phases (Metges*, et al.*, 1996). However, irrespective of the which procedure is used for acetylation, during the esterification process the N atom in the side groups of glutamine and asparagine is lost, converting them to their respective acids. As a result, the measured isotopic values are mixed values from glutamine/glutamate and aspargine/aspartate.

The reaction of amino acids with ECF (in presence of ethanol and pyridine) to their respective N(O,S)-ethoxycarbonylethyl esters (Figure 7) is directly compatible with GC-C-IRMS analysis. It was reported for the first time fairly recently (Husek, 1991a; b) and has also been used for amino acids and organic acids simultaneously (Husek, 1995). The derivatization is a one-step procedure and high yields are reported for most amino acids. It also introduces a low number of carbon atoms (5) to the derivative. Very recently, Sacks and Brenna (2005) took nitrogen CSIA a step further when they used the ECF reaction for site-specific $\delta^{15}N$ analysis of polynitrogenous amino acids following enzymatic or acid hydrolysis.

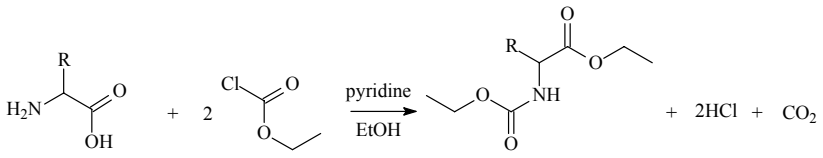

Figure 7. The ECF derivatization scheme.

Glycine is one of the simplest amino acids to derivatize, with high yields reported for all the techniques described above. ECF was used in Paper **IV** for derivatization of glycine due to its simplicity and also because of the small number of carbon atoms introduced. Nordin (1998) suggests that amino acids other than glycine should preferably also be included in plant uptake studies since glycine has only been found to be the dominating amino acid in alpine soil. The ECF derivatization technique can be used for all amino acids found in proteins except arginine, for which the imino group remains untouched during derivatzation and consequently the molecule does not elute from the gas chromatographic columns used for amino acids (Husek, 1991b). Hence, the use of ECF makes it possible to extend the method in Paper **IV** to also cover other amino acids present in soil. By using the optically active menthyl chloroformate for derivatization, separation of amino acid enantiomers was possible (Domergue *et al.*, 1993).

Another aspect to keep in mind when derivatizing for GC-C-IRMS analysis is the relatively high detection limits compared to other gas chromatographic detectors, such as FID, electron capture detection (ECD), and conventional

organic MS. Standard derivatization procedures are generally scaled for the amounts needed for detection with other techniques and their capacity might therefore be insufficient for the amounts required in GC-C-IRMS. As an example, the ECF procedure described by Husek (1991b) for FID detection is not recommended for use with samples containing more than 100 µg of amino acids (≈ 0.7 µmole) in total, giving a molar ratio of ECF to amino acids of approximately 75:1. The ECF procedure of Husek (1991b) is therefore only suitable for preparing samples for analysis by GC-C-IRMS if one or possibly two amino acids are to be derivatized and no other reagent-consuming compounds are present in the sample. For the derivatization of glycine in Paper **IV**, the quantity of reagents was increased to enable analysis of up to approximately 14 µmoles (assuming a 75:1 ratio to be the lower limit). The low recoveries obtained for most amino acids following different derivatization techniques applied to standard amino acid solutions reported by Hofmann*, et al.* (2003) can probably be explained by the low molar ratio (< 30:1) between the limiting reagent and analytes.

Kinetic isotopic fractionation can occur during the derivatization procedure, if one of the reagents reacts non-quantitatively. However, in nitrogen analysis, an isotopic fractionation can occur only if the *analyte* reacts non-quantitatively, since the reactions involve no incorporation of nitrogen from the reagents. When using optimal derivatization conditions, the reagents should be present in excess of the compound(s) of interest and the reaction should proceed to completion, or at least close to completion. In ^{13}C analysis, the risk of kinetic fractionation is larger, due to incorporation of carbon from the reagents. Rieley (1994) discussed these effects, as well as showed calculations of how to get the true ^{13}C value from derivatized compound.

Separation

Baseline separation of chromatographic peaks is critical in GC-C-IRMS in order to obtain high precision isotopic ratios. Complex curve fitting procedures have been successfully applied to separate unresolved peaks mathematically (Goodman and Brenna, 1994), but these procedures have yet to be incorporated into commercially available software packages for interpretation of IRMS chromatograms.

The properties of the separation column are the most important determinants of peak resolution quality, and as a rule of thumb the polarity of the separation column should be similar to the polarity of the analytes. Furthermore, the temperature program and the gas flow rates should be adjusted and optimized for the application in question. Also, a retention gap can be used to focus the analytes after injection to give sharper peaks.

The solvent in which the analytes are present will quickly consume the oxygen present in the oxidation column and also plug the cold trap when injected into a GC-C-IRMS system. Hence, the carrier gas flow is directed towards an FID through a switchable valve at the beginning of each run (see Figure 3). Deng *et al.* (2004) used SPME for extraction of amino acids derivatized with a chloroformate

prior to GC-MS analysis. It was unclear if the SPME extraction *per se* resulted in a lower detection limit compared to normal solvent injection, but this approach is nevertheless interesting for GC-C-IRMS analysis since it completely avoids the injection of a solvent.

Isotope Dilution Mass Spectrometry

Isotope dilution mass spectrometry (IDMS) is a technique based on addition of a spike (a compound with known concentration and isotopic composition) to a sample containing the same compound (of different isotopic composition), followed by measurement of the isotopic ratio in the mixture. IDMS is conventionally used for high precision quantitative determinations by measuring the isotopic ratio in the sample before and after addition of the spike and is considered to be a primary method of measurement (Berglund, 2004). Briche *et al.* (2000) used the IDMS technique when establishing new reference materials for ethanol, using GC-C-IRMS for determination of $\delta^{13}C$.

The ratio between the amounts of the native (x) and spike (y) can be expressed as:

$$\frac{x}{y} = \frac{\left(R_y - R_m\right) * \left(R_x + 1\right)}{\left(R_m - R_x\right) * \left(R_y + 1\right)} \tag{9}$$

where R_x, R_y and R_m are the isotopic ratios of the native compound, spike and mixture, respectively (i.e from Colby *et al.* (1981)).

IDMS can also be used to measure the isotopic ratio in a compound, provided the concentration is known (measured by an independent technique), an approach that can be used if the analyte concentration is not high enough for isotopic measurement. The ratio of the analyte in the native pool, R_x, can be calculated after a rearrangement of Eq 9:

$$R_x = \frac{\left(R_y - R_m\right) - R_m\left(R_y + 1\right)\frac{x}{y}}{\left(R_m - R_y\right) - \left(R_y + 1\right)\frac{x}{y}} \tag{10}$$

With an additional step in the analytical procedure, the total measurement uncertainty is increased. Uncertainty budgets for the IDMS technique can be calculated according to Kragten (1994) and Briche*, et al.* (2000) to get an indication of the parameter(s) limiting the total uncertainty. Generally, the precision obtained is not sufficient for natural abundance studies, but well suited for tracer studies.

In Paper **IV** this approach was used to extend the working range for the nitrogen isotopic ratio estimate of free glycine in a soil extract downwards. It was shown that the nitrogen ratio of the native glycine pool could be estimated with a relative uncertainty in the AP value of between 3 and 15 %. The precision obtained is

generally higher than the precision obtained with conventional GC-MS and the technique can also be used for lower enrichment levels.

The uncertainty in the measured R_m was generally the source dominating the uncertainty in R_x. Also, the uncertainty in the estimation of the very low native amount of glycine (x, by HPLC) made a substantial contribution to the total uncertainty. Clearly, by minimizing the measurement uncertainty, a more precise R_x value will be obtained. For a specific application, an approximate uncertainty can be calculated to evaluate if the precision is sufficiently high for the experiment, even without performing any laboratory experiments.

Concluding Remarks and Future Perspectives

During recent decades, the use of nitrogen isotopes in different tracer studies, as well as in natural abundance studies, within ecological research has been increasing. This is due to the development of automated high precision continuous flow IRMS instruments, which have extended the working range downwards, as well as increased the sample throughput. This thesis is mainly focused on sample preparation for the analysis of specific nitrogen compounds, namely ammonium and glycine, from soils prior to compound specific isotope analysis by GC-C-IRMS. Their very low concentration in soils has been shown to constitute the largest problem for analysis.

For ammonium analysis, three alternative methods have been presented. The direct equilibrium headspace method (Paper **I**) is easily performed with today´s commercially available GC-C-IRMS systems and showed high precision in the isotope analysis. The sensitivity with the experimental settings tested in Paper **I** was not sufficient for most soil extracts, but with some modifications, including a large-volume injector and a cold trap, it can potentially be used for solutions containing N in the low mg L^{-1} range. The use of custom-made SPME fibre coatings for ammonia (Paper **II**) slightly increased the sensitivity compared to the direct headspace technique, but at the expense of precision. In addition, the Nafion® fibre used for absorption showed a memory effect which affected the measured isotopic value of the next sample. The P & T technique also showed a memory effect during desorption. In addition, the measured isotopic ratio was largely affected by the size of the memory effect, an effect which was difficult to control.

The development of new materials for use in sample preparation for isotopic ratio measurements is not straightforward. Memory effects (found for Nafion® in Paper **II** and the sulphonated sty/DVB copolymer in Paper **III**) and isotopic discrimination are two phenomena which can occur. Further research is needed regarding suitable materials for the preparation of samples for ammonium analysis. SPME and P & T are two techniques which are of particular interest for use with GC-C-IRMS, since it does not involve injection of a solvent and at the same time accomplishes a pre-concentration of the analyte(s).

Due to the relatively simple procedure and the inexpensive equipment used in diffusion of ammonia followed by EA-IRMS analysis, it is anticipated that this technique will probably attain dominance in the future. The problems reported when diffusing large sample volumes makes the detection limit of the EA-IRMS instrumentation critical. Successful attempts have been made to extend the working range for these types of instruments (Fry*, et al.*, 1996), but commercialisation is needed for a general break-through.

Also for amino acids, the nitrogen isotope ratio determination is limited by the low concentrations in soil samples. In Paper **IV** of this thesis, high precision estimates of isotopic ratios for glycine were possible for soil extracts containing only 3 μM (0.042 mg N L^{-1}) as the native concentration, using a combination of sample pre-concentration and the isotope dilution mass spectrometry technique. The precision is not enough to be used with natural abundance studies, but with the use of a more sensitive instrumentation, which exists on the market, the IDMS step could probably have been omitted and the precision improved. IDMS can be used when the instrumentation, in combination with sample preparation is insufficient for the actual sample concentrations (Paper **IV**). *A priori* measurement uncertainty estimates can give information on whether or not the precision which can be obtained is sufficient for the specific application.

Alongside improvements of IRMS instruments performed by different manufacturers, such as the introduction of GC-C-IRMS systems, there are also improvements which can be made in the laboratory. For example, two papers from 1998 report significant improvements in chromatographic resolution when using a single capillary interface for oxidation by reducing band broadening (Ellis and Fincannon, 1998; Goodman, 1998). In nitrogen analysis, the designs of the reduction column and the CO_2-trap are also important and could potentially be improved.

The use of GC-C-IRMS will for sure also increase in the future, as the number of measurable analytes and hence the fields of application increase. However, for each analyte/matrix combination, the different sample preparation and analysis steps need to be carefully evaluated for isotope effects, as shown for ammonium and glycine in this thesis.

Literature Cited

Adamsen, F.J. and Reeder, J.D. 1983. A Comparison of Diffusion and Distillation for Preparing Samples for ^{15}N Analyses. *Soil Science Society of America Journal 47,* 612-613.

Aguilar-Cisneros, B.O., Lopez, M.G., Richling, E., Heckel, F. and Schreier, P. 2002. Tequila Authenticity Assessment by Headspace SPME-HRGC-IRMS Analysis of $^{13}C/^{12}C$ and $^{18}O/^{16}O$ Ratios of Ethanol. *Journal of Agricultural and Food Chemistry 50,* 7520-7523.

Ahad, J.M.E., Sherwood Lollar, B., Edwards, E.A., Slater, G.F. and Sleep, B.E. 2000. Carbon Isotope Fractionation During Anaerobic Biodegradation of Toluene: Implications for Intrinsic Bioremediation. *Environmental Science and Technology 34,* 892-896.

Amelung, W. and Zhang, X. 2001. Determination of Amino Acid Enantiomers in Soils. *Soil Biology & Biochemistry 33,* 553-562.

Arthur, C.L. and Pawliszyn, J. 1990. Solid Phase Microextraction with Thermal Desorption Using Fused Silica Optical Fibers. *Analytical Chemistry 62,* 2145-2148.

Barraclough, D. 1997. The Direct or MIT Route for Nitrogen Immobilization: A N-15 Mirror Image Study with Leucine and Glycine. *Soil Biology & Biochemistry 29,* 101-108.

Barrie, A. and Prosser, S.J. 1996. Automated Analysis of Light-Element Stable Isotopes by Isotope Ratio Mass Spectrometry. In *Mass Spectrometry of Soils*, *Vol. 1.* pp. 1-46. eds. Boutton, T.W. and Yamasaki, S., Marcel Dekker Inc., New York, NY.

Bartels, J.M. (ed.) 1996. *Methods of Soil Analysis. P. 3, Chemical Methods*, Soil Science Society of America Book Series. Madison, Wisconsin. 1390 pp.

Beneteau, K.M., Aravena, R. and Frape, S.K. 1999. Isotopic Characterization of Chlorinated Solvents-Laboratory and Field Results. *Organic Geochemistry 30,* 739-753.

Bengtsson, G. and Bergwall, C. 2000. Fate of ^{15}N Labelled Nitrate and Ammonium in a Fertilized Forest Soil. *Boil Biology and Biochemistry 32,* 545-557.

Berglund, M., Introduction to Isotope Dilution Mass Spectrometry (IDMS). In *Handbook of Stable Isotope Analytical Techniques*, *Vol. 1.* pp. 820-834., ed. de Groot, P.A., Elsevier B.V., Amsterdam, The Netherlands.

Blau, K. and Halket, J. (eds.) 1993. *Handbook of Derivatives for Chromatography*, 2nd ed., John Wiley & Sons Ltd. Chichester, UK. 369 pp.

Brand, W.A., Tegtmeyer, A.R., and Hilkert, A. 1994. Compound-Specific Isotope Analysis: Extending toward $^{15}N/^{14}N$ and $^{18}O/^{16}O$. *Organic Geochemistry 21,* 585-594.

Bremner, J.M. and Keeney, D.R. 1966. Determination and Isotope-Ratio Analysis of Different Forms of Nitrogen in Soils: 3. Exchangeable Ammonium, Nitrate and Nitrite by Extraction-Distillation Methods. *Soil Science Society of America Proceedings 30,* 577-582.

Briche, C., Hernandez, H., O'Connor, G., Webb, K.S. and Catterick, T. 2000. High Accuracy Isotope Dilution Analysis for the Determination of Ethanol Using Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry. *Analyst 125,* 2189-2195.

Brooks, P.D., Stark, J.M., McInteer, B.B. and Preston, T. 1989. Diffusion Method to Prepare Soil Extracts for Automated Nitrogen-15 Analysis. *Soil Science Society of America Journal 53,* 1707-1711.

Buresh, R.J., Austin, E.R., and Craswell, E.T. 1982. Analytical Methods in ^{15}N Research. *Fertilizer Research 3,* 37-62.

Caimi, R.J. and Brenna, T.J. 1993. High-Precision Liquid Chromatography-Combustion Isotope Ratio Mass Spectrometry. *Analytical Chemistry 65,* 3497-3500.

Colby, B.N., Rosecrance, A.E. and Colby, M.E. 1981. Measurement Parameter Selection for Quantitative Isotope Dilution Gas Chromatography/Mass Spectrometry. *Analytical Chemistry 53,* 1907-1911.

Dasgupta, P.K. and Dong, S. 1986. Solubility of Ammonia in Liquid Water and Generation of Trace Levels of Standard Gaseous Ammonia. *Atmospheric Environment 20,* 565-570.

Davidson, E.A., Hart, S.C., Shanks, C.A. and Firestone, M.K. 1991. Measuring Gross Nitrogen Mineralisation, Immobilization and Nitrification by ^{15}N Isotopic Pool Dilution in Intact Cores. *Journal of Soil Science 42,* 335-349.

Dayan, H., Abrajano, T., Sturchio, N.C. and Winsor, L. 1999. Carbon Isotopic Fractionation During Reductive Dehalogenation of Chlorinated Ethenes by Metallic Iron. *Organic Geochemistry 30,* 755-763.

Deng, C.H., Li, N. and Zhang, X.M. 2004. Rapid Determination of Amino Acids in Neonatal Blood Samples Based on Derivatization with Isobutyl Chloroformate Followed by Solid-Phase Microextraction and Gas Chromatography/Mass Spectrometry. *Rapid Communications in Mass Spectrometry 18,* 2558-2564.

Dias, R.F. and Freeman, C.H. 1997. Carbon Isotope Analyses of Semivolatile Organic Compounds in Aqueous Media Using Solid-Phase Microextraction and Isotope Ratio Monitoring GC/MS. *Analytical Chemistry 69,* 944-950.

Domergue, N., Pugniere, M. and Previero, A. 1993. One-Step Conversion of Amino Acids into N-Menthyloxycarbonyl Alkyl Ester Derivatives for Chiral Gas Chromatography. *Analytical Biochemistry 214,* 420-425.

Ellis, L. and Fincannon, A.L. 1998. Analytical Improvements in irm-GC/MS Analyses: Advanced Techniques in Tube Furnace Design and Sample Preparation. *Organic Geochemistry 29,* 1101-1117.

Fry, B., Garritt, R., Tholke, K., Neill, C., Michener, R.H. and Mersch, F.J. 1996. Cryoflow: Cryofocusing Nanomole Amounts of CO_2, N_2 and SO_2 from an Elemental Analyzer for Stable Isotope Analysis. *Rapid Communications in Mass Spectrometry 10,* 953-958.

Fujihara, S., Nakashima, K. and Kurogochi, Y. 1986. Determination of $^{15}NH_3$ by Gas Chromatography - Mass Spectrometry. Applicaion to the Measurement of Putrescine Oxidation by Human Plasma. *Journal of Chromatography 383,* 271-280.

Gebel, G. 2000. Structural Evolution of Water Swollen Perfluorosulfonated Ionomers from Dry Membrane to Solution. *Polymer 41,* 5829-5838.

Giesler, R., Lundström, U.S. and Grip, H. 1996. Comparison of Soil Solution Chemistry Assessment Using Zero-Tension Lysimeters or Centrifugation. *European Journal of Soil Science 47,* 395-405.

Gilbert, R.G. and Altman, J. 1966. Ethanol Extraction of Free Amino Acids from Soil. *Plant and Soil 24,* 229-238.

Goodman, K.J. and Brenna, J.T. 1994. Curve Fitting for Restoration of Accuracy of Overlapping Peaks in Gas Chromatography/Combustion Isotope Ratio Mass Spectrometry. *Analytical Chemistry 66,* 1294-1301.

Goodman, K.J. 1998. Hardware Modifications to an Isotope Ratio Mass Spectrometer Continuous-Flow Interface Yielding Improved Signal, Resolution and Maintenance. *Analytical Chemistry 70,* 833-837.

Górecki, T., Martos, P. and Pawliszyn, J. 1998. Strategies for the Analysis of Polar Solvents in Liquid Matrixes. *Analytical Chemistry 70,* 19-27.

Goupry, S., Rochut, N., Robins, R.J. and Gentil, E. 2000. Evaluation of Solid-Phase Microextraction for the Isotopic Analysis of Volatile Compounds Produced During Fermentation by Lactic Acid Bacteria. *Journal of Agricultural and Food Chemistry 48,* 2222-2227.

Gray, J.R., Lacrampe-Couloume, G., Gandhi, D., Scow, K.M., Wilson, R.D., Mackay, D.M. and Sherwood Lollar, B. 2002. Carbon and Hydrogen Isotopic Fractionation During Biodegradation of Methyl *tert*-Butyl Ether. *Environmental Science and Technology 36,* 1931-1938.

Hales, J.M. and Drewes, D.R. 1979. Solubility of Ammonia in Water at Low Concentrations. *Atmospheric Environment 13,* 1133-1147.

Hall, J.A., Barth, J.A.C. and Kalin, R.M. 1999. Routine Analysis by High Precision Gas Chromatography Mass Selective Detector Isotope Ratio Mass Spectrometry to 0.1 Parts Per Mil. *Rapid Communications in Mass Spectrometry 13,* 1231-1236.

Handley, L.L. and Raven, J.A. 1992. The Use of Natural Abundance of Nitrogen Isotopes in Plant Physiology and Ecology. *Plant, Cell and Environment 15,* 965-985.

Hauk, R.D., Meisinger, J.J. and Mulvaney, R.L. 1994. Practical Considerations in the Use of Nitrogen Tracers in Agricultural and Environmental Research. In *Methods of Soil*

Analysis, Part 2. *Microbiological and Biological Properties*, pp. 907-950. ed. Mickelson, S.H., Soil Science Society of America. Madison, WI.

Hofmann, D., Jung, K., Segschneider, H.J., Gehre, M. and Schüürmann, G. 1995. $^{15}N/^{14}N$ Analysis of Amino Acids with GC-C-IRMS - Methodological Investigations and Ecotoxicological Applications. *Isotopes in Environmental and Health Studies 31,* 367-375.

Hofmann, D., Gehre, M. and Jung, K. 2003. Sample Preparation Techniques for the Determination of Natural $^{15}N/^{14}N$ Variations in Amino Acids by Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS). *Isotopes in Environmental and Health Studies 39,* 233-244.

Holmes, R.M., McClelland, J.W., Sigman, D.M., Fry, B. and Peterson, B.J. 1998. Measuring $^{15}NH_4^+$ in Marine, Estuarine and Fresh Waters: An Adaptation of the Ammonia Diffusion Method for Samples with Low Ammonium Concentrations. *Marine Chemistry 60,* 235-243.

Huang, L., Sturchio, N.C., Abrajano, T.J., Heraty, L.J. and Holt, B.D. 1999. Carbon and Chlorine Isotope Fractionation of Chlorinated Aliphatic Hydrocarbons by Evaporation. *Organic Geochemistry 30,* 777-785.

Hunkeler, D. and Aravena, R. 2000. Determination of Compound-Specific Carbon Isotope Ratios of Chlorinated Methanes, Ethanes, and Ethenes in Aqueous Samples. *Environmental Science and Technology 34,* 2839-2844.

Husek, P. 1991a. Amino Acid Derivatization and Analysis in 5 Minutes. *FEBS Letters 280,* 354-356.

Husek, P. 1991b. Rapid Derivatization and Gas-Chromatographic Determination of Amino Acids. *Journal of Chromatography 552,* 289-299.

Husek, P. 1995. Simultaneous Profile Analysis of Plasma Amino and Organic Acids by Capillary Gas Chromatography. *Journal of Chromatography B 669,* 352-657.

Högberg, P. 1997. Tansley Review No 95 - N-15 Natural Abundance in Soil-Plant Systems. *New Phytologist 137,* 179-203.

Ivarson, K.C. and Sowden, F.J., 1969. Free Amino Acid Composition of the Plant Root Environment under Field Conditions. *Canadian Journal of Soil Science 49,* 121-127.

Johnston, A., Scrimgeour, C., Kennedy, H. and Handley, L. 2003. Isolation of Ammonium-N as 1-Sulfonato-Iso-Indole for Measurement of $\delta^{15}N$. *Rapid Communications in Mass Spectrometry 17,* 1099-1106.

Jones, D.L., Owen, A.G. and Farrar, J.F. 2002. Simple Method to Enable the High Resolution Determination of Total Free Amino Acids in Soil Solutions and Soil Extracts. *Soil Biology & Biochemistry 34,* 1893-1902.

Kelley, K.R., Ditsch, D.C. and Alley, M.M. 1991. Diffusion and Automated Nitrogen-15 Analysis of Low-Mass Ammonium Samples. *Soil Science Society of America Journal 55,* 1016-1020.

Kirshenbaum, I., Smith, J.S., Crowell, T., Graff, J. and KcKee, R. 1947. Separation of the Nitrogen Isotopes by the Exchange Reaction between Ammonia and Solutions of Ammonium Nitrate. *The Journal of Chemical Physics 15,* 440-446.

Kolb, B. and Ettre, L.S. 1997. *Static Headspace - Gas Chromatography. Theory and Practice.* Wiley-VCH. New York. 298 pp.

Koopal, L.K., Yang, Y., Minnaard, A.J., Theunissen, P.L.M. and Van Riemsdijk, W.H., 1998. Chemical Immobilisation of Humic Acid on Silica. *Colloids and Surfaces A: Physiochemical and Engineering Aspects 141,* 385-395.

Kragten, J. 1994. Calculating Standard Deviations and Confidence Intervals with a Universally Applicable Spreadsheet Technique. *The Analyst 119,* 2161-2165.

Krummen, M., Hilkert, A.W., Juchelka, D., Duhr, A., Schluter, H.J. and Pesch, R. 2004. A New Concept for Isotope Ratio Monitoring Liquid Chromatography/Mass Spectrometry. *Rapid Communications in Mass Spectrometry 18,* 2260-2266.

Lauf, J. and Gebauer, G. 1998. On-Line Analysis of Stable Isotopes of Nitrogen in NH_3, NO and NO_2 at Natural Abundance Levels. *Analytical Chemistry 70,* 2750-2756.

Lehmann, M.F., Bernasconi, M. and McKenzie, J.A. 2001. A Method for the Extraction of Ammonium from Freshwaters for Nitrogen Isotope Analysis. *Analytical Chemistry 73,* 4717-4721.

Lindau, C.W. and Spalding, R.F. 1984. Major Procedural Discrepancies in Soil Extracted Nitrate Levels and Nitrogen Isotopic Values. *Ground Water 22,* 273-278.

Liu, S.R. and Dasgupta, P.K. 1995. Liquid Droplet - a Renewable Gas Sampling Interface. *Analytical Chemistry 67,* 2042-2049.

Macko, S.A., Uhle, M.E., Engel, M.H. and Andrusevich, V. 1997. Stable Nitrogen Isotope Analysis of Amino Acid Enantiomers by Gas Chromatography Combustion / Isotope Ratio Mass Spectrometry. *Analytical Chemistry 69,* 926-929.

Mariotti, A. 1983. Atmospheric Nitrogen is a Reliable Standard for Natural ^{15}N Abundance Measurements. *Nature 303,* 685-687.

Maris, C., Laplanche, A., Morvan, J. and Bloquel, M. 1999. Static Headspace Analysis of Aliphatic Amines in Aqueous Samples. *Journal of Chromatography 846,* 331-339.

Matthews, D.E., and Hayes, J.M. 1978. Isotope-Ratio-Monitoring Gas Chromatography-Mass Spectrometry. *Analytical Chemistry 50,* 1465-1473.

Meier-Augenstein, W., Brand, W., Hoffmann, G.F. and Rating, D. 1994. Bridging the Information Gap between Isotope Ratio Mass Spectrometry and Conventional Mass Spectrometry. *Biological Mass Spectrometry 23,* 376-378.

Meier-Augenstein, W. 1995. On-Line Recording of $^{13}C/^{12}C$ Ratios and Mass Spectra in One Gas Chromatographic Analysis. *Journal of High Resolution Chromatography 18,* 28-32.

Meier-Augenstein, W. 1997. The Chromatographic Side of Isotope Ratio Mass Spectrometry - Pitfalls and Answers. *LC-GC 15,* 244-253.

Meier-Augenstein, W. 2004. GC and IRMS Technology for ^{13}C and ^{15}N Analysis on Organic Compounds and Related Gases. In *Handbook of Stable Isotope Analytical Techniques*, *Vol. 1.* pp. 157-176., ed. de Groot, P.A., Elsevier B.V., Amsterdam, The Netherlands.

Merritt, D.A. and Hayes, J.M. 1994. Nitrogen Isotopic Analyses by Isotope-Ratio-Monitoring Gas-Chromatography Mass-Spectrometry. *Journal of the American Society for Mass Spectrometry 5,* 387-397.

Metges, C.C., Petzke, K.-J. and Hennig, U. 1996. Gas Chromatography / Combustion / Isotope Ratio Mass Spectrometric Comparison of N-Acetyl- and N-Pivaloyl Amino Acid Esters to Measure ^{15}N Isotopic Abundances in Physiological Samples: A Pilot Study on Amino Acid Synthesis in the Upper Gastro-Intestinal Tract of Minipigs. *Journal of Mass Spectrometry 31,* 367-376.

Metges, C.C. and Petzke, K.-J. 1997. Measurement of ^{15}N / ^{14}N Isotopic Composition in Individual Plasma Free Amino Acids of Human Adults at Natural Abundance by Gas Chromatography Combustion Isotope Ratio Mass Spectrometry. *Analytical Biochemistry 247,* 158-164.

Miyajima, T., Yamada, Y. and Hanba, Y.T. 1995. Determining the Stable Isotope Ratio of Total Dissolved Inorganic Carbon in Lake Water by GC/C/IRMS. *40,* 994-1000.

Monreal, C.M. and McGill, W.B. 1985. Centrifugal Extraction and Determination of Free Amino Acids in Soil Solutions by TLC Using Tritiated 1-Fluoro-2,4-Dinitrobenzene. *Soil Biology & Biochemistry 17,* 533-539.

Mook, W.G. 2002. Environmental Isotopes in the Hydrological Cycle-Principles and Applications. Volume 1: Introduction- Theory, Methods, Review. UNESCO/IAEA Series. pp. 31-48.

Morrill, P.L., Lacrampe-Couloume, G. and Sherwood Lollar, B. 2004. Dynamic Headspace: A Single-Step Extraction for Isotopic Analysis of mg L^{-1} Concentrations of Dissolved Chlorinated Ethenes. *Rapid Communications in Mass Spectrometry 18,* 595-600.

Mulvaney, R.L. 1993. Mass Spectrometry. In *Nitrogen Isotope Techniques*, pp. 11-55., eds. Knowles, R. and Blackburn, T.H., Academic Press., San Diego, California.

Nordin, A. 1998. *Physiological Ecology of Nitrogen Utilisation by Forest Plants.* Doctoral Thesis. Swedish University of Agricultural Sciences, Department of Forest Genetics and Plant Physiology, Umeå.

Nordin, A., Högberg, P. and Näsholm, T. 2001. Soil Nitrogen Form and Plant Nitrogen Uptake Along a Boreal Forest Productivity Gradient. *Oecologia 129,* 125-132.

Näsholm, T., Ekblad, A., Nordin, A., Giesler, R., Högberg, M. and Högberg, P. 1998. Boreal Forest Plants Take up Organic Nitrogen. *Nature 392,* 914-916.

O´Deen, W.A., Porter, L.K. 1979. Digestion Tube Diffusion and Collection of Ammonia for Nitrogen-15 and Total Nitrogen Determination. *Analytical Chemistry 51,* 586-589.

O´Deen, W.A., Porter, L.K. 1980. Devarda´s Alloy Reduction of Nitrate and Tube Diffusion of the Reduced Nitrogen for Indophenol Ammonium and Nitrogen-15 Determinations. *Analytical Chemistry 52,* 1164-1166.

Odham, G. and Bengtsson, G. 1987. Microanalysis of Amino Acids by GC/ECD and GC/MS: Applications in Ecological and Medical Research. In *Amino Acid Analysis by Gas Chromatography*, *Vol. 3.* pp. 65-91, eds. Zumwalt, R., Kuo, K.C.T. and Gehrke, C.W., CRC Press. Boca Raton, Florida.

Ostle, N.J., Bol, R., Petzke, K-.J. and Jarvis, S.C. 1999. Compound Specific $\delta^{15}N$ Values: Amino Acids in Grassland and Arable Soils. *Soil Biology & Biochemistry 31,* 1751-1755.

Paul, E.A. and Schmidt, E.L. 1960. Extraction of Free Amino Acids from Soil. *Soil Science Society of America Proceedings 24,* 195-198.

Persson, J. and Näsholm, T. 2001. A GC-MS Method for Determination of Amino Acid Uptake by Plants. *Physiologia Plantarum 113,* 352-358.

Preston, T. and Owens, N.J.P. 1983. Interfacing an Automatic Elemental Analyser with an Isotope Ratio Mass Spectrometer: The Potential for Fully Automated Total Nitrogen and Nitrogen-15 Analysis. *The Analyst 108,* 971-977.

Preston, T. and Slater, C. 1994. Mass Spectrometric Analysis of Stable-Isotope-Labelled Amino Acid Tracers. *Proceedings of the Nutrition Society 53,* 363-372.

Preston, T., Bury, S., Presing, M., Moncoiffe, G. and Slater, C. 1996. Isotope Dilution Analysis of Combined Nitrogen in Natural Waters. I. Ammonium. *Rapid Communications in Mass Spectrometry 10,* 959-964.

Prosser, S.J. 1993. A Novel Magnetic Sector Mass Spectrometer for Isotope Ratio Determination of Light Gases. *International Journal of Mass Spectrometry and Ion Processes 125,* 241-266.

Rieley, G. 1994. Derivatization of Organic Compounds Prior to Gas Chromatographic-Combustion-Isotope Ratio Mass Spectrometric Analysis: Identification of Isotope Fractionation Processes. *The Analyst 119,* 915-919.

Sacks, G.L. and Brenna, J.T. 2005. $^{15}N/^{14}N$ Position-Specific Isotopic Analyses of Polynitrogenous Amino Acids. *Analytical Chemistry 77,* 1013-1019.

Saghir, N.S., Mulvaney, R.L., Azam, F. 1993. Determination of Nitrogen by Microdiffusion in Mason Jars. I. Inorganic Nitrogen in Soil Extracts. *Communications in Soil Science and Plant Analysis 24,* 1745-1762.

Scrimgeour, C.M. 1995. Measurement of Plant and Soil Water Isotope Composition by Direct Equilibration Methods. *Journal of Hydrology 172,* 261-274.

Silfer, J.A., Engel, M.H., Macko, S.A. and Jumeau, E.J. 1991. Stable Carbon Isotope Analysis of Amino Acid Enantiomers by Conventional Isotope Ratio Mass Spectrometry and Combined Gas Chromatography / Isotope Ratio Mass-Spectrometry. *Analytical Chemistry 63,* 370-374.

Simpson, I.A., Bol, R., Bull, I.D., Evershed, R.P., Petzke, K.-J. and Dockrill, S.J. 1999. Interpreting Early Land Management through Compound Specific Stable Isotope Analyses of Archaeological Soils. *Rapid Communications in Mass Spectrometry 13*, 1315-1319.

Slater, G.F., Dempster, H.S., Sherwood Lollar, B. and Ahad, J. 1999. Headspace Analysis: A New Application for Isotopic Characterization of Dissolved Organic Contaminants. *Environmental Science and Technology 33,* 190-194.

Slater, G.F., Preston, T. and Weaver, L.T. 2001. Stable Isotopes and the International System of Units. *Rapid Communications in Mass Spectrometry 15,* 1270-1273.

Sommer, S.G. and Olesen, J.E. 2000. Modelling Ammonia Volatilization from Animal Slurry Applied with Trail Hoses to Cereals. *Atmospheric Environment 34,* 2361-2372.

Sowden, F.J. and Ivarson, K.C. 1966. The "Free" Amino Acids of Soil. *Canadian Journal of Soil Science 46,* 109-114.

Stark, J.M. and Hart, S.C. 1996. Diffusion Technique for Preparing Salt Solutions, Kjeldahl Digests and Persulfate Digests for Nitrogen-15 Analysis. *Soil Science Society of America Journal 60,* 1846-1855.

Sörensen, P. and Jensen, E.S. 1991. Sequential Diffusion of Ammonia and Nitrate from Soil Extracts to a Polytetrafluroethylene Trap for ^{15}N Determination. *Analytica Chimica Acta 252,* 201-203.

Teffera, Y., Kusmierz, J.J. and Abramson, F.P. 1996. Continuous-Flow Isotope Ratio Mass Spectrometry Using the Chemical Reaction Interface with Either Gas or Liquid Chromatographic Introduction. *Analytical Chemistry 68,* 449-456.

Thode, H.G., Graham, R.L., and Ziegler, J.A. 1945. A Mass Spectrometer and the Measurement of Isotope Exchange Factors. *Canadian Journal of Research 23B,* 40-47.

Ward, J.A.M., Ahad, J.M.E., Lacrampe-Couloume, G., Slater, G.F., Edwards, E.A. and Sherwood Lollar, B. 2000. Hydrogen Isotope Fractionation During Methanogenic Degradation of Toluene: Potential for Direct Verification of Bioremediation. *Environmental Science and Technology 34,* 4577-4581.

Zwank, L., Berg, M., Schmidt, T.C. and Haderlein, S.B. 2003. Compound-Specific Carbon Isotope Analysis of Volatile Organic Compounds in the Low-Microgram Per Liter Range. *Analytical Chemistry 75,* 5575-5583.

Acknowledgements

This thesis work was performed as a co-operation between the Department of Forest Ecology, SLU and the Department of Analytical Chemistry, Umeå University and was mainly financed by the Centre for Environmental Research in Umeå (CMF). Financial support given from Professor Peter Högberg (SLU; funding for strategic research) is gratefully acknowledged. The Soil Science Laboratory, Department of Forest Ecology provided expertise and laboratory infrastructure for analytical research on stable isotope methods for soil science. Travel grants from the S.M. Kempe´s Foundation, Knut and Alice Wallenberg Foundation ("Jubileumsdonationen") and "SLU-medel för internationalisering av forskarutbildningen" are gratefully acknowledged, as well as financial support for analytical equipment from the Mauritz Carlgren Foundation.

I would like to thank my principal supervisor Anders Ohlsson for support during the whole process. You have always had time to listen, discuss and comment on my ideas and manuscripts. I especially appreciate all the time you spent on me and my work during the last intensive months. Thanks are also due to Peter Högberg, who was my principal supervisor at the beginning of the project and who contributed with ecological perspectives on the work. My co-supervisor from Umeå University, Knut Irgum, is thanked for providing his time when I needed it. Your knowledge in analytical chemistry in general and polymer chemistry in particular has been invaluable.

Duc Nguyen is thanked for a huge contribution to Paper III and also my lab mate Håkan Wallmark for help in all kinds of situations, as well as nice discussions about music and life in general. Since last year Birgitta Olsson and Bengt Andersson have also contributed to the nice atmosphere in the lab. I would also like to thank my former office mate Yu Chen for good co-operation when working with the Nafion® experiments. I have probably consumed 1000 litres of liquid nitrogen during my lab work. Thank you Bertil Wiberg at vaktmästeriet for providing it, always on short notice.

Emma Tilston is gratefully acknowledged for improving the English in the summary of the thesis.

Past (Ylva L, Gert, Ulrik, Jin, Zewdu, Johan S, Johan E, Hjalmar, Mona and Tong) and present (Andreas, Tobias, Tesfay, Zhulan, Ylva W, Kajsa, Sofia, Ishi, Sabine, Per, Jörgen, Ylva S, Lena, Yu, Torbjörn, Stina, Zacharia and Korodjouma) PhD students at the Department of Forest Ecology are thanked for their co-operation during teaching commitments, encouragement, nice conversations, lunches, and social activities (even though I have not been especially social the last year). Good luck to all of you who have your dissertation ahead of you! Also, all other colleagues at the Department of Forest Ecology are thanked for these years.

My “old” bosses Einar Pontén (SeQuant) and Robert Hansson (AlControl) made me believe in myself, which I think is a prerequisite for completing a PhD project. Thank you Lars Lundmark for letting me perform my master’s thesis in Laos, a trip which meant a lot to me. Also, our working trips to Russia have been very nice.

All my friends from sports, folk music gatherings, travels, “baby activities” (including all the nice children) and other social activities are deeply thanked for making my life rich. You are too many to be mentioned but I would like you to know that you are all important to me.

Helena, my old friend from school, thank you for our friendship. I really appreciate our weekly phone calls and, at least annual meetings. Twelve years have past since we made our inter-rail trip in Europe, where we happened to meet two PhDs on a night train. I think we made a deal (?). Here is my thesis, have you prepared your speech?!

The Svensson/Wingfors family is thanked for support and encouragement during the years. My parents, sister and brother– thank you for believing in me and supporting me in different ways. Berit and my mother are especially thanked for helping us a lot with Linnéa during the last year.

Finally, some words to my beloved family. Håkan, my love, thank you for always supporting me. Now it is time to spend more time together!! Linnéa, my little daughter, thank you for always meeting me with a smile. You really bring in a new perspective in my life!

Umeå, August 2005.

Elin