Isotope ratio mass spectrometry in forensic science applications

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HIGHLIGHTS

• Review of forensic applications of isotope ratio mass spectrometry publications since 2009.
• Includes details on good practices for calibration, quality assurance and uncertainty assessments and reporting.
• Review is organized into 10 different forensic science disciplines.

ABSTRACT

This review highlights advances in the application of isotope ratio mass spectrometry (IRMS) to materials of interest to the forensic community. We give special attention to the natural abundance differences in the stable isotopes of carbon, nitrogen, hydrogen and oxygen because they are the most abundant and informative elements in typical forensic casework. We also present some of the recent developments in normalization, error reporting and data analysis, which together provide a more scientific and robust foundation for admissibility in court. The review focuses on research published in the last decade and is organized into 10 major sections, including human provenancing, wildlife forensics, environmental forensics, seized drugs, ignitable liquids, explosives, food forensics, poisoning, questioned documents and miscellaneous applications.

1. Introduction

The ability to unravel the source of chemically indistinguishable substances has made isotope ratio mass spectrometry (IRMS) an indispensable tool in a variety of scientific disciplines, and especially in forensic science [1–3]. There are many excellent reviews and monologs on the theory, development, and application of stable isotope analysis (SIA) in different forensic science areas of study [4–13]. Herein, we do not attempt to be so comprehensive. Instead, we focus on the most recent, novel and impactful forensic applications, including new aspects of data analysis and reporting. In the last decade, IRMS, in combination with improved chromatographic techniques, has provided advances in diverse areas of forensic science, including sample preparation techniques that have enabled more reliable or meaningful discrimination between samples. The review is organized into ten major topical areas of forensic interest. Most of the citations are for applications that involve source provenance and sample classification, but some articles report on better understanding the fractionation of isotopes within or between certain substrates.

Since its foundation in 2002, the Forensic Isotope Ratio Mass Spectrometry Network (FIRMS) has spearheaded the application of IRMS to forensic casework. FIRMS has been organizing triennial conferences, providing forensic practitioner approvals, conducting inter-laboratory proficiency tests [2,14], presenting guidance on suitable reference materials (RMs), and offering a “Good Practice Guide for IRMS” (GPG) to assist practitioners with the essential principles of the instrument operation, quality assurance, troubleshooting and data management [15]. The FIRMS Network often publishes a special issue in an analytical or forensic science journal following each triennial conference, and these special editions are a rich source of forensically-relevant IRMS literature for anyone interested in the topic.

Relating to the FIRMS Network, Carter and Fry [16] conducted a series of inter-laboratory tests within FIRMS Network members to first assess the variance, quality control and quality assurance protocols of different laboratories, and then to guide improvements in the reproducibility of measurements between laboratories. Problems related to homogeneity in sample preparation, choice of reference materials and internal standards, as well as data reporting relative to delta (δ) scale, are among the identified deficiencies that required improvements to make IRMS results fit-for-purpose. Only a minority of labs were outside the expected range of the results. More recently, another inter-laboratory assessment was performed to compare the bulk isotope
analysis of honey coming from diverse metrological institutes [17]. The results derived from forensic stable isotope laboratories within the FIRMS Network [18], and all the participating laboratories, provided δ13C values for bulk honey that were within an acceptable range. The method by which each laboratory calculated and reported their measurement uncertainty was a notable point of discussion, however, because some laboratories report standard deviations instead of expanded measurement uncertainty, such as the 95% confidence interval. To align with international standards, the use of an expanded uncertainty instead of a standard deviation is recommended for reported results.

To assist practitioners in establishing expanding uncertainties, Dunn and coworkers [19] have suggested a simple and straightforward improvement to the Kragnet spreadsheet published in the GPG [15], which complements the existing approaches and guidelines used for delta scale [20]. The new spreadsheet is quite practical, and it combines the different sources of isotope uncertainty to multiple samples in one spreadsheet. Note that analytical results can include human errors, which are outside the random errors that are captured in uncertainty-budgeting spreadsheets. All researchers and practitioners should take preventative steps to promptly identify and mitigate sources of occasional mistakes, thereby enhancing the reliability of any reported result [21,22].

One of the biggest challenges in IRMS is the selection and use of appropriate—i.e. matrix matched—and well-characterized reference materials (RMs) [23]. The last few years have witnessed significant developments in RMs for calibration of δ2H, δ18O [24–27], δ13C, δ15N, and δ34S values [28–30]. One RM is a mixture of three glycine solutions that are recommended for establishing multiple-point calibration curves for the δ13C scale [29]. Another three sugar RMs (BEET-1, GALT-1 and FRUT-1) were developed for δ13C normalization of sugar-based materials, although it can also be used for other organic materials [31]. The hair RMs USGS-42 and USGS-43 have been developed for measurements of hydrogen and oxygen stable isotopes, but are also appropriate for measurements involving C, N, and S of human and mammalian hair [28]. The use of (at least) a two-point calibration scale is now a minimum requirement for high-quality data [15,23,32,33], and procedures are available with which to provide in-house isotope standards to help preserve the limited stocks of international materials [34].

Beyond the importance of accuracy, it is also important to report measures of precision or confidence intervals for forensic applications and to provide estimates for the power of discrimination of forensic samples [35]. As examples of these approaches, several groups have used Bayesian networks to assess the value of IRMS data [36–38], and several groups have provided a method using likelihood ratios (LRs) as a measure of the strength of IRMS data [39–41]. Such developments in reporting are critical to the future of the forensic community. However, one potential problem with reporting results in terms of LRs is that when an LR weakly favors the prosecution, the LR can be totally misinterpreted by jurors as actually favoring the defense [42]! Another way to interpret this observation is that jurors demand a very high threshold of confidence to find a suspect guilty, so a weakly favorable LR might not pass the cognitive threshold in the jurors' minds to warrant a severe penalty for the defendant. For a contextual understanding of the use of IRMS in legal applications, Ehleringer and Matheson have provided an excellent, comprehensive, clear review article that describes the admissibility of stable isotope analyses in court [43]. This foundational review is a profoundly important document for bridging the gap between the legal community and the research/practitioner communities, and there are many other forensic science disciplines that could benefit from such lucid a foundational document.

2. Human provenance

Many forensic stable isotope studies involve human subjects, and the goal of such investigations is usually to provide an investigative lead, such as insight into a person’s geographic origin or travel history. Assisting such investigations, many IRMS studies have examined the relationship between the isotopic composition of human tissues and the geographic origin, health status or dietary interventions of the subjects. Such studies are useful for both paleo-archaeological purposes and modern law enforcement investigations because both applications require the prediction of geographic origin of unidentified human remains [10,11,44–46].

A critical factor for strong geo-location inference is the establishment of reliable and high-resolution spatial maps for the precipitation and groundwater values for δ2H and δ18O [47,48]. Such isotopic landscapes, also known as Isoscapes [49], provide the framework for spatiotemporal movement of humans and other organisms [2,47,50]. In 2014, δ2H and δ18O measurements of 349 US tap water samples were made available in the largest public dataset to date. The samples included a broad geographic sampling and seasonal sampling [51]. Dr. Gabriel Bowen, from the University of Utah, has made the spatial variation metadata for water isotopes fully accessible through the Water Isotopes Database (http://wateriso.utah.edu/waterisotopes/index.html) and through a recently developed mobile phone application called “wiSamples”. Publicly-available data like this are critical for the validation and acceptance of Isoscapes for modern human provenancing, and some groups have successfully started using Isoscapes for such applications [52]. As a word of caution to prospective researchers in this area, one has to be especially careful with the calibration and interpretation of δ2H measurements of proteinaceous material like hair, muscle and feathers [53]. The number of exchangeable hydrogens varies greatly depending on the protein composition, so it is critical to use matrix-matched standards to obtain accurate isotope ratio normalization [54].

For geographical provenance determinations, biological samples must be representative of the organism and be chemically robust to provide meaningful isotope ratios, which is why bone, teeth, hair and nails are especially useful matrices. Hair and fingernails have the added advantages that they store a chronological record of diet and health of a person and that they can be non-invasively collected in the presence of a third party; i.e. witnessed [55,56]. Hair and nails are both abundant in α-keratin—a protein that is extremely robust and insoluble in water under normal conditions. As an example of keratin’s stability, a study by Koehler and Hobson showed that tanning polar bear hides with sulfuric acid did not alter the δ13C or δ15N values of the hair in the hides but did alter the δ34S values [57]. In another example, isotope ratio values of δ13C, δ15N in human hair were shown to be equally stable in a desiccator and freezer as they were spending ten months exposed to the natural environment [58]. However, as cautioned above, some δ2H fractionation of hair was observed after prolonged storage times (e.g. 6 months). The general, chemical stability of keratins means that once they are formed, metabolic and chemical changes are minimal, and the isotopes of C, N and O are resistant to external influence.

A global database of δ13C and δ15N in hair and nails was recently compiled [59], and whereas the δ13C values of hair and fingernails were shown to correlate strongly with the amount of C4 plants in the diet—as determined by latitude and geography—the δ15N values of hair and fingernails showed a much weaker correlation to geography. Instead, the δ15N values correlated more strongly with fish/meat intake and coastal proximity (Fig. 1). Valenzuela et al. showed that within the United States the δ34S values of more than 206 human hair samples provided better geographic information than either δ13C or δ15N [60]. However, the samples in this particular study were almost devoid of coastal representation, which is where dietary factors are most likely to influence the δ13C and δ15N values.

Although most of the literature related to human provenancing focuses on the dietary components and geospatial movement of individuals [10,11], non-dietary sources of phenotypic variance, such as sex, BMI and age, also enable the classification of subjects when
Fig. 1. Example of a global isotope database against which questioned samples can be compared. This map shows the spatial distribution of δ\(^{13}\)C values collected from more than 4,000 contemporary human hair and nail samples. Equatorial regions, especially on the African continent, are vastly under-represented. Image reprinted from reference [59]: F. Hulsemann, C. Lehn, S. Schneider, G. Jackson, S. Hill, A. Rossmann, N. Scheid, P.J. Dunn, U. Flenker, W. Schanzer, Global spatial distributions of nitrogen and carbon stable isotope ratios of modern human hair, Rapid Commun. Mass Spectrom. 29(22) (2015) 2111–21, Copyright (2015), with permission from John Wiley & Sons, Inc.

Performing compound-specific isotope analysis (CSIA) of hair [61]. One note of caution with hair and fingernail comparisons is that human hair is slightly enriched in \(^{13}\)C and slightly depleted in \(^{15}\)N relative to fingernail clippings from the same individual [62–64], so care must be taken when interpreting the isotope values between hair and nails.

3. Wildlife forensics

Studies involving illegal harvesting and the black-market trading of plants and animals are of great interest in the legal community. Illegal wildlife trade is a global problem estimated to circulate US$5–20 billion annually [65]. The determination of the geographic origin and movement of animals between different landscapes have both been successfully tracked using IRMS. For example, different isotopes are often used to unravel the trophic level of organisms and to establish the various flows of energy inside different food webs [66]. Such studies support a better understanding of the ecological behavior of animals and the development of effective conservation strategies towards endangered species [67]. IRMS can also assist with wildlife crime investigations [67]. Even though IRMS can be very relevant in these particular forensic situations, there is a formal delineation that divides forensic applications from "pure" ecological investigations. As with other cross-disciplinary studies, there are many benefits to maintaining an open dialog between forensic science and ecology application areas.

Carbon, nitrogen and, to a lesser extent, sulfur are the most studied isotopes for diet reconstruction, geographic origin determination, and the study of animal migration/foraging ecology in different habitats [68–72]. These applications also lead to a better understanding of illegal trading of wildlife products. Among these applications, elephant ivory and bone were the first wildlife materials where original source determination via multi-isotope analysis was combined with a forensic application [73–76]. More recently, δ\(^{13}\)C and δ\(^{15}\)N values were used to discriminate between the breeding of wild and captive Vietnamese endangered crocodile lizards of the taxon *Shinisaurus crocodilurus* [77]. SIA was also successful in determining the differences between wild vs. illegally traded/captive-bred African grey parrots (*Psittacus erithacus*) [78] and Burmese and reticulated pythons [79]. However, carbon and nitrogen trophic models are not trivial markers of energy flow through an ecosystem because of isotope fractionation. The term fractionation refers to the enrichment or depletion of isotope ratios when a substrate undergoes a physical or chemical transformation. Fractionation occurs when a bond containing a heavier isotope is involved in a rate-limiting chemical transformation, such as when an enzyme decarboxylates an amino acid during metabolism. The heavier isotope has a slightly higher bond dissociation energy than the lighter isotope, so the rate of chemical transformation of the heavier isotope is slightly slower, which leads to the preferential transformation of the lighter light isotope [80,81].

Although measurable, isotopic fractionation between an organism and its food sources is not always predictable, especially in some highly dynamic and complex ecosystems like aquatic food webs [66]. For this reason, some researchers use isoscapes [82], and \(^{87}\)Sr/\(^{86}\)Sr isotope ratios in addition to δ\(^{13}\)C and δ\(^{15}\)N values to predict an organism’s movements. Such studies provide a better understanding of how SIA can be applied for wildlife conservation practices. Extra caution must be taken to ensure that the results are fit-for-purpose and devoid of fractionation [53,54]. Meanwhile, when comparing high-resolution X-ray fluorescence (XRF) and δ\(^{13}\)C and δ\(^{15}\)N values of wild- and zoo-bred specimens of echidnas (*Tachyglossus aculeatus*), Brandis et al. reported 100% correct classification using the XRF data compared to 91.3% accuracy from the IRMS data [83]. Such studies are a reminder that IRMS measurements are not always the best or only tool in the analytical chemist’s toolbox.

In the context of aquatic systems, some studies have investigated the influence of dietary water on the δ\(^{2}H\) values of different organisms’ tissues, as well as how water signatures can be used to trace organic matter and migratory patterns [66,84,85]. For example, Soto and coworkers [86] performed an environmentally controlled experiment where the ambient water, dissolved O\(_2\) food and tissue protein and lipids of two trophic level aquatic species—an insect and a fish—were...
analyzed for $\delta^2$H and $\delta^{18}$O values. The authors demonstrated that hydrogen and oxygen could serve as complementary tracers of diet and provenance in the aquatic ecosystem and pointed out the strengths and weaknesses of this approach. The lipid content of animal tissue is often reported as a source of variation in $\delta^2$H, $\delta^{13}$C and $\delta^{15}$N values from aquatic animals’ tissues [84,87–89]. Independent of the organism, the removal of lipids prior to bulk tissue analysis for their separated analysis or a posterior mathematical lipid normalization is a general recommendation in stable isotope ecology [86,89,90]. However, Patterson and Carmichael recently suggested that lipid extraction may actually not be necessary for all taxa, but more important in gycogen storing species such as the Eastern oyster Crassostrea virginica [91]. This overall lipid correction practice should be reviewed in a species-specific and tissue-specific manner to avoid unnecessary sample handling and misinterpretation of isotopic values in trophic ecology studies. In fact, Connan et al.’s recent study with fish samples showed that different pre-treatments widely used to remove interfering biological molecules (lipids, urea, TMAO) affect the isotopic data and suggested a more standardized pre-treatment approach to ensure the comparison and reproducibility of bulk SIA [92].

For terrestrial environments, the use of $\delta^2$H and $\delta^{18}$O analysis of tissues is not always tightly correlated with the precipitation $\delta^2$H and $\delta^{18}$O isoscapes, which might make geographic determinations more challenging. An example was reported with two North American carnivores, the bobcat (Lynx rufus) and the puma (Puma concolor), wherein both $\delta^2$H and $\delta^{18}$O values from hair samples of these felines lacked correlation with local isoscapes [93]. This study highlights the importance of additional investigations into isotopic fractionation and metabolic routing if IRMS is to find a stronger footing in wildlife forensics.

In recent work at the boundary between human provenancing and wildlife forensics, two reports have investigated the light stable isotope composition of blowflies and their diets [94,95]. Both studies showed isotope fractionation between the flesh and the blow flies that was significantly smaller than the isotopic variance that existed between the food sources, thereby enabling the discrimination of potential food sources. Through CSIA of amino acids in the different life stages of blow flies, the study by Matos et al. demonstrated that essential and non-essential amino acids are fractionated differently in the different life stages of the blow flies [95].

Regarding forest studies, Thomas et al. analyzed $\delta^{13}$C and $\delta^{34}$S values from red cedar (Juniperus virginiana L.) tree rings and presented a recent and gradual recovery of this species from the decades-long acidic pollution that affected the Appalachian region [96]. Despite the direct correlation with forensics, this work revealed the effectiveness of stricter environmental legislation on human activity and also showed the potential of IRMS in forest ecosystems research.

Timber traceability coupled to initiatives against deforestation and illegal logging is another area that can strongly benefit from the use of stable isotope fingerprinting. The combination of $\delta^{15}$N, $\delta^{34}$S, $\delta^{18}$O values, $^{87}$Sr/$^{86}$Sr ratios, and radiocarbon dating was successful in identifying relocated South African cycad species removed from the wild, which supports the use of SIA as a prospective method to combat the illegal trading of endangered populations [97]. However, different isotopes have different inferential value depending on the species in question. For wood sourcing, or dendroprovenancing, of two Pinyon pines species in the southwestern United States, the $\delta^{13}$C values from the tree rings proved to be a more precise and successful method than the analysis of ring widths by itself [98]. However, when working with Norway spruces (Picea abies Karst.) in and around the European Alps, carbon isotopes did not work as a proxy for their geolocation. In contrast, $\delta^2$H and $\delta^{18}$O values were more suitable for European spruces [99]. Stable isotopes have also been successful proxies in archaeological wood sourcing investigations [100], and could even be used for chronology determination of tropical trees with indistinct annual rings [101].

### 4. Environmental forensics

Environmental forensics covers a broad range of topics and involves the illegal contamination or damage of protected areas, whether intentional or not. A typical example would be the use of $\delta^{13}$C values to...
determine the point source of an environmental pollutant. Due to the complexity that arises when investigating the source or fate of a contaminant release, it is usually necessary to use a variety of analytical techniques to resolve the issue, as shown in Table 1 [102]. Compound-specific isotope ratio analysis of δ13C, δ15N, δD, δ18O, δ37Cl, δ34S, and δ7Br have been comprehensively documented [103-107] alongside the advances and inherent challenges when applied to the micropollutants [108]. IRMS is mostly used in studies involving anthropogenic contamination because most pollutants are man-made and contain an isotope profile that differs significantly from natural sources of the same substance. Examples include chemical leaks and oil spills, which pose a major hazard to marine and coastal environments.

In a weathering simulation experiment of a simulated oil spill, GC-C-IRMS measured the δ13C values of individual n-alkanes (C_{12}-C_{33}) in crude oil and showed that δ13C values are generally unaltered by short-term weathering [109]. The lack of fractionation during mild weathering makes δ13C analysis a suitable tool for oil fingerprinting such complex samples [110]. However, fractionation of hydrocarbons does occur in simulated fire debris samples, so the effects of elevated temperature, pyrolysis and combustion must be better understood before reliable inferences can be made between post-combustion residues and pre-combustion liquids from which they derived [111].

5. Seized drugs

As stated in the 2013 National Survey on Drug Use and Health (NSDUH) report [125], approximately 24.6 million American people aged 12 or more self-declared illicit drug use in the previous year. Given the extent of novel psychoactive substances (NPSs) appearing on the market, IRMS has emerged as a potential technique to infer the cultivation location of drugs of plant origin, to establish trafficking routes, and to establish the synthetic pathways and chemical lots used in clandestine laboratories.

Cannabis sativa L., also known as cannabis or marijuana, is the most common plant-derived illicit drug in the United States and the world [126]. There were approximately 19.8 million users in the US in 2013 [125], and its use has apparently skyrocketed since the legalization of medical and recreational marijuana in some US states [127]. With the additional announcement that the White House has alleviated restrictions on medical marijuana studies in some US states [128], scientists now have more legal support to conduct research in this area. For forensic purposes, IRMS has been mostly applied to infer cannabis source and distribution networks. Most of the data on large-scale drug trafficking samples is collected by the DEA special testing laboratory and it is unclear how much of the available data makes it to the public domain. This is not to criticize the DEA, but to highlight the point that the public domain probably does not contain all that has been learned about the isotopic composition of drug seizures from around the world.

Carbon and nitrogen stable isotopes are the most well-explored isotopes for the origin determination and growth conditions assessment of Cannabis seized in different countries [129-131], and the DEA’s database provides insight into trafficking, production and distribution networks [132]. Based on the differences in bulk δ13C and δ15N values from leaves and inflorescences of 554 marijuana samples, West et al. suggested an isotopic framework range to interpret the isotope ratio values [133]. An example is shown in Fig. 2. By combining parameters of indoor or outdoor growth and inorganic or organic fertilizer, West et al. demonstrated a linkage between isotopes and possible cultivation method beyond the correct geographical assignment of known seized samples [126,134]. In a complementary work, Tipple and coworkers found that the study of chain length distributions, concentrations and δ13C values of n-alkanes (n-C_{20}) from seized Cannabis inflorescences can also provide information about the cultivation settings of marijuana plants [135].

When characterizing marijuana samples seized in Alaska, Booth et al. demonstrated that multiple isotopes (δ13C, δ15N, δD and δ18O) can be a useful approach to trace trafficking patterns [136], whereas other authors are exploring the 87Sr/86Sr ratios retained in marijuana leaves and inflorescences to achieve a similar goal [137]. A subsequent publication from the Jackson group described the comparison of bulk δ13C values of Cannabis plant samples to compound-specific δ13C values of cannabinoids extracted from the same samples [138]. Although the advanced age of some of the samples had caused some isomerization and degradation of the cannabinoids, the results effectively showed that individual cannabinoids have unique δ13C values relative to the bulk plant matter, which therefore makes it somewhat challenging to link extracted THC to a particular plant source.

Among all plant-derived drugs, cocaine’s production and trafficking routes around the world are of particular interest to law enforcement agencies. In this context, SIA appears to be well-suited to the task because seized cocaine samples closely reflect the growth environment of coca plants. Mallette and coworkers [139] applied a multiple bulk isotope approach (δ13C, δ15N, δD and δ18O) combined with trace alkanoids and multivariate statistics to discriminate cocaine coming from 19 major South American growing regions. A similar method was used to differentiate and determine the unique profile of the first illicit coca plantation in Mexico, showing that coca cultivation for cocaine production is expanding its limits outside South America [140]. Under controlled laboratory conditions, those same isotopes were investigated regarding their fractionation patterns in fractionally precipitated cocaine base. Interestingly, the authors observed an opposite trend to the usual Rayleigh fractionation because the earlier fractions of precipitated samples were more depleted than the later ones for all four isotopes. 13N and 2H were characterized by the largest fractionations [141].

γ-hydroxybutyric acid (GHB) is another illicit psychoactive drug that became very popular among young people at nightclubs or raves because of the desirable increase in euphoria and disinhibition. GHB use also provides less-desirable effects, including amnesia, which also made GHB attractive for use in drug-facilitated sexual assaults. The popularity caught the authorities’ attention because GHB, or its chemical precursors γ-butyrolactone (GBL) or 1,4-butanediol (1,4-BD) are colorless and odorless liquids, which makes them easy to spike into beverages of intended victims of drug-assisted sexual assault. Marclay and coworkers used GC/C-IRMS to investigate the effects of in vivo metabolism on δ13C values of GHB [142]. Urinary GHB δ13C values ranged from -25.06‰ to -24.81‰ for patients who had ingested prescribed pharmaceutical GHB, which was indistinguishable from the original δ13C value of -24.99‰. Given that metabolism of GHB had no statistical influence on the isotopic ratio, comparison of a seized exogenous GHB and endogenous GHB from a victim can be used as a successful approach to incriminate/acquit a sexual assault suspect. In much the same way, IRMS can distinguish endogenous vs. exogenous sources of performance-enhancing hormones in athletes. Marclay and coworkers had previously used the same instrument to determine the source of 19 GBL samples obtained worldwide by measuring intra and inter-variability of their bulk carbon isotope signatures [143].

In other applications with synthetic drugs, IRMS elucidated different production batches of 23 seized tablets of the pipеразине analogs benzylpiperazine (BZP) and trifluoromethylphenylpiperazine (TFMPP) [144], both synthetic stimulants sold as alternatives to ecstasy. In a parallel study, the same group reported the IRMS analysis three batches of benzylpiperazine hydrochloride (BZP-HCl) and its synthetic intermediates, which were synthesized from three different precursor suppliers [145]. Although all the intermediate samples were significantly different and correctly associated with their respective precursor suppliers, the increased variance in the measured δ13C and δ15N values for BZP-HCl meant that not all the BZP-HCl batches could be uniquely linked to the correct precursor suppliers. IRMS has also been used in combination with other profiling techniques to classify carfentanil seizures [146].

A comprehensive discussion on the application of IRMS to
methylamphetamine (also known as methamphetamine) and its precursors has already been provided by Gentile et al. [7] and will not be repeated in detail here. In another review, Collins and Salouros highlight the strategic importance of $\delta^{13}C$, $\delta^2H$ and $\delta^{15}N$ analysis as a complementary profiling technique to impurity profiling for the routine analysis of methylamphetamine seizures [12,147]. Fig. 3 demonstrates the range of $\delta^{13}C$ and $\delta^2H$ values obtained for 782 samples seized at the Australian border and the ability to distinguish semi-synthetic from natural or synthetic methylamphetamine. Other valuable work by Liu et al. compared the isotope ratios of ephedra plants, natural ephedrine, synthetic ephedrine and the partially- or totally-synthetic methamphetamine products [148]. Although the results enabled inferences to be made about the likely source of 987 methamphetamine casework samples, the ground truth for each casework sample was not known, so the casework-specific false positive and false negative rates could not be assessed.

6. Ignitable liquids

According to the National Fire Protection Association (NFPA), around 282,600 intentional fires per year were reported to U.S. fire departments between 2007 and 2011, including 211,500 outside or unclassified fires, 50,800 structure fires and 20,400 vehicle fires [149]. Approximately 160,910 were found to be intentionally set with an ignitable liquid [150]. In spite of these statistics, and in contrast to the extensive use of IRMS on explosives, very few manuscripts have reported the analysis of ignitable liquids with IRMS [7].

Since 2009, diesel fuel has become a more frequently studied ignitable liquid [151–153] even though gasoline is the most commonly used ignitable liquid in arson cases [150]. In one study, Harvey et al. first isolated the n-alkanes from different diesel samples to ensure baseline resolution between each n-alkane and prevent any potential interference between peaks [151]. A following combination of $\delta^2H$ and $\delta^{13}C$ CSIA with multivariate statistics was then used to distinguish between the four diesel samples of different sources. Muhammad et al. examined a larger dataset of 45 diesel samples from various gas stations in New Zealand [152]. This study showed the difficulty of source attribution when there is only one major supplier of diesel to all the gas stations in the region. Source attribution was scientifically accurate, but of little forensic value.

Weathering and additives are the possible reasons for subtle differences in fuel isotopic compositions. By analyzing the fingerprint pattern of n-alkanes (e.g. $nC_{12}$ to $nC_{23}$) via GC-IRMS, Muhammad et al. reaffirmed the discriminant power of CSIA. Moreover, following 21 days of evaporation at room temperature (24 ± 2°C), hydrogen and carbon isotopic signatures of various n-alkanes in a diesel sample [153],
the n-alkane underwent negligible fractionation for $^{12}\text{C}$ and varying degrees of fractionation of $^2\text{H}$. For example, long-chain alkanes were not fractionated during evaporation, but the shorter chain alkanes $\text{nC}_{12}$–$\text{nC}_{17}$ underwent significant $^2\text{H}$ fractionation.

Several groups have also reported evaporation effects on isotope ratios of different petroleum derivatives [154–156]. Whereas IRMS appears to be a suitable tool to predict potential common sources of pristine flammable liquid samples, Schwartz and coworkers showed that post-combustion residues in simulated fire debris undergo unpredictable extents of $^{12}\text{C}$ fractionation ranging from 0 to +10% relative to the same analytes in the pristine, unburned liquid [111]. The absence of any reliable trend in fractionation in simulated fires makes IRMS unsuitable for the comparison of pre-combustion compounds and their post-combustion residues. Not only do the analytes undergo fractionation during evaporation and pyrolysis, but pyrolysis products from the organic substrate (e.g. carpet) also contribute different weighted $^{13}\text{C}$ values to different residues [157].

7. Explosives

Source determination of explosives has become a growing interest in a variety of government sectors, especially because of the daily use of a broad range of explosive devices in military actions, terrorism and insurgents’ attacks. Recently, a combination of IRMS and inductively coupled plasma–mass spectrometry (ICP–MS) was used to provide a better discrimination between ammonium nitrate (AN) samples from distinct AN batches and manufacturers [158]. The low cost and easy access of AN as a fertilizer ensures that ammonium nitrate fuel oil (ANFO), consisting of a mixture of 94% AN and 6% fuel oil, is possibly still one of the most used materials employed in improvised explosive devices (IEDs) [159]. Brust et al. showed that $^{15}\text{N}$ and $^{18}\text{O}$ isotopic signatures of AN samples could successfully distinguish between the different samples [158]. Carbon and hydrogen analyses were also performed, but low sample carbon contents and an observed influence of nitrogen on the $^2\text{H}$ values made these two isotopes less useful. Benson et al. [160] achieved similar results when the source of Australian AN samples was differentiated based on their $^2\text{H}$, $^13\text{O}$, and $^{15}\text{N}$ values [161].

Some challenges in working with nitrogen-rich organic compounds have been reported by other researchers. For example, incomplete reduction might cause the tailing in the $\text{N}_2$ peak of nitrate [162]. Caution must also be used when measuring organic substances against inorganic reference materials like IAEA-N1 and IAEA-N2 [162]. In addition, nitrogen-rich compounds can also influence the accuracy and precision of $^2\text{H}$ isotope analysis [163]. Given that these compounds undergo thermochemical processes that are different from typical organic compounds in EA-IRMS, and that they tend to give lower than expected recoveries of nitrogen, Gentile et al. recommend a thermal decomposition method to remove of any external oxygen [162]. Additional results to this premise indicate that, compared to the usual EA combustion method, thermal decomposition provides more precise $^{13}\text{N}$ and $^{15}\text{N}$ results for both organic and inorganic materials containing oxidized nitrogen. The thermal decomposition procedure did not have a deleterious effect on the measurement of $^{15}\text{N}$, $^{13}\text{C}$ or $^{15}\text{N}$ values of materials containing reduced nitrogen [164]. Adjustments to this methodology would allow a more accurate, faster and cheaper way to analyze $^{13}\text{N}$ materials using EA-IRMS. Other manuscripts have described that isotope analysis of each of the ion components of nitrogen-rich explosives (such as ammonium nitrate or urea nitrate) provides additional discriminatory power for the investigation of forensic samples [165–167].

Using samples synthesized in different conditions, Benson et al. presented the use of IRMS to differentiate triacetone triperoxide (TATP) samples, another common explosive in many terrorist bombings [168]. Based on bulk $^{13}\text{C}$ values, the samples were classified according to their synthetic process. Carbon, hydrogen, and oxygen isotopic data were combined to classify the TATP samples to their common source. A global study by Howa et al. showed that $^{13}\text{C}$ values of TATP correlate strongly with the $^{13}\text{C}$ values of locally-available liquid acetone, the synthetic precursor to TATP [169].

Using bulk $^{13}\text{C}$ and $^{15}\text{N}$ values from a sample set containing the explosive pentaerythritol tetranitrate (PETN), Benson et al. showed the discrimination of PETN from detonating cord or cast PETN booster filling [168]. Howa and coworkers [170] investigated the factors that influence the carbon and nitrogen isotopic ratios of commercial PETN by isolating the explosive from the bulk sample of different manufacturers and observing the isotopic linkage between that substance and its reactants, pentaerythritol (PE) and nitric acid. The results showed that, whereas $^{13}\text{C}$ values from PETN reflect the PE carbon signatures, PETN $^{15}\text{N}$ values are influenced by both nitric acid $^{15}\text{N}$ values and the deflagration reaction parameters. The authors suggested further experiments with controlled reaction conditions to provide a better understanding of the results.

The effectiveness and limitations of IRMS were also reported in studies involving RDX, HMX, TNT, HMTD, black powders and other pre- and post-blast materials from explosive devices [113,171–176]. Whereas bulk analyses like these are extremely helpful for linking evidence in pre-detonation scenarios, there are significantly fewer reports linking post-detonation residues with pre-detonation samples. The CSIA of individual analytes in multi-component explosives would be helpful to connect the explosive precursor to the product samples. In this context, Chesson and coworkers provide a very useful step-by-step approach for the extraction and preparation of several explosive mixtures for IRMS (Fig. 4) [177]. The same group established a robust database that helps establish the use of explosive isotopic evidence in court [178].

8. Food forensics

Since the 1990s, the application of SIA in several food adulteration/safety studies has been recognized as an official method by AOAC International [179]. The use of isotopic techniques has also become prominent among the practiced methods to authenticate and protect citizens and businesses against a variety of legal issues involving foods and brands.

IRMS can provide insight regarding authenticity, provenance, and contamination of foodstuffs, and it has been used in a variety of legal cases [180]. Although counterfeit food is not always dangerous for consumption, counterfeiting is illegal and strongly affects the consumer confidence on the product or brand [181]. Food fraud is most prevalent when products are expensive to produce like premium-brand products. In such cases, black-market suppliers will typically employ cheaper, lower-quality ingredients and less labor-intensive manufacturing methods to produce the final product.

Honey is a commonly counterfeited product. Carbon bulk isotope analysis has been used for the detection of commercial honey adulteration (AOAC–C4 Sugar Method 998.12) [182] because most authentic honey has a $^{13}\text{C}$ value close to C4 plants. However cheap substitutes include syrup sugars made from corn or sugar cane, which shift the $^{13}\text{C}$ values towards C4 plants values [183,184]. The AOAC method identifies this adulteration because it requires the extraction of protein from honey and compares the differences between $^{13}\text{C}$ values of honey protein vs. bulk honey ($^\Delta^{13}\text{C}$). Differences greater than 1% indicate that the honey has more than 7% adulteration with a C4 syrup [185,186]. However, elemental analysis by bulk IRMS is not very effective when honey is diluted with cheap C4-plant-derived sugars like sugar beet because the bulk isotope ratio would not be substantially different [186,187]. One exception to the rule is the premium-priced New Zealand manuka honey, which naturally has a high level of C4 sugar, and therefore has an isotope ratio that is indistinguishable from cane or corn syrup [188]. Because bulk IRMS cannot always identify adulteration in honey, LC-IRMS can provide more selectivity by
providing compound-specific carbon isotope ratio measurements [189,190]. Furthermore, the use of multi-isotope approaches to classify honey according to their botanical and/or geographical origin has also been reported (Fig. 5) [191,192].

Zhao et al. [193] and Camin et al. [180,181] have both published thorough reviews about the applications of IRMS in authenticity and traceability of a variety of plant-based and animal-based foods. Another very comprehensive reading on the topic can be found in [194]. Isotope analysis has also contributed to the tracking of animal diets for the protection of premium products as well as for consumer health reasons. IRMS has been used to protect against bovine spongiform encephalopathy (BSE), foot-and-mouth disease (FMD), and even avian influenza [193]. Most studies involve a multiple isotope approach and sometimes include additional analytical techniques. Applications include the determination of the primary diets of poultry [195–198], pigs [199,200], beef [201], lamb [202,203] and dairy products [204]. Other examples of food applications include the geographic origin of meat [205,206], fish and shellfish [207–211], dairy [212–215], vegetables and fruits [216–219], grain-based food [220–225], chocolate [226], coffee [227], alcoholic and nonalcoholic drinks [228–233] and oils [234–237]. The influence of local ingredients on “global” foods, such as Big Mac® patties collected in 26 countries was also accessed via isotope analysis. The δ13C values of hamburgers reflected the diets of the local cattle [238,239].

Fig. 4. Suggested workflow to isolate and prepare different components from explosive mixtures for measurement by IRMS and other analytical techniques. Image reprinted from reference [177]: L.A. Chesson, J.D. Howa, M.J. Lott, J.R. Ehleringer, Development of a methodological framework for applying isotope ratio mass spectrometry to explosive components, Forensic Chem. 2 (2016) 9–14, Copyright (2016), with permission from Elsevier.
Food fraud is a dynamic activity, however, and it poses a continuous challenge for researchers. Beyond the common use of $\delta^{13}$C values for food authenticity and $\delta^{15}$N values for the distinction of crops raised with organic or synthetic fertilizers, some groups are already incorporating the use of hydrogen and oxygen isotopes as valuable markers for geographic determination. For example, when investigating 436 commonly consumed meat samples from diverse terrestrial and marine animals, Chesson et al. reported that the correlations between $\delta^2$H and $\delta^{18}$O values from animal tissues reflect the consumed water similarly, independent of the animal taxa [240]. Hydrogen and oxygen isotopes were also applied to demonstrate that hamburgers sold at local restaurants are more likely to come from regionally raised cattle, while fast food chains rely on beef coming from further regions [13]. Interestingly, most food forensic articles apply some quite advanced chemometrics, which indicates a gradual change in the isotope community towards a more sophisticated interpretation of results.

The effect of cooking on the isotope ratios of foodstuff is not commonly investigated, although many consumed items in a human/animal diet are cooked before consumption. It was recently reported that $\delta^{13}$C and $\delta^{15}$N values of grain-based desserts and yeast bread are not affected by baking or fermentation procedures, and the raw ingredients from processed food can be used to estimate the respective isotopic ratio values [241]. Other research found that steaming, grilling and boiling of two types of fish (mackerel and haddock) did not have a significant effect on the bulk of two types of fish (mackerel and haddock) did not have a significant effect on the bulk $\delta^{13}$C values for the distinction of crops raised with organic or synthetic fertilizers. For this reason, $\delta^{15}$N analysis alone is not a very suitable parameter for organic authentication [258,259]. A combination of multiple isotopes, multivariate statistics, and other analytical techniques is therefore preferred. Successful multivariate approaches have included IRMS with ICP-MS [257] and IRMS with mid-infrared spectroscopy, (MIS) and Proton Nuclear Magnetic Resonance ($^1$H NMR) [260] to classify organic vs. conventionally-grown tomatoes. In addition, CSIA has also demonstrated good discriminatory power between plants that are grown under different conditions [258,259,261].

Besides all the benefits listed above, there are still some limitations for IRMS in practical food applications. The high costs to maintain IRM instrument are well known, but the lack of an extensive reference database containing products coming from a range of different diets, production conditions, locations and seasons is possibly the biggest problem [181]. Such a database would also have to be continually updated because of the dynamic nature of global food markets. To our knowledge, wine is the only product with a well established isotope database, which was created in the early 1990s in the European Union [262]. Finally, one challenge in relating food forensics to forensic casework, such as nutritional studies of humans, is that even when controlled studies examine the consumption of large quantities of a certain food, like beer, the isotope signature of the intervention food group is so diluted by the natural variation in a person’s diet that the dietary intervention may not be observable [263].

9. Poisoning

Although not as common as food authentication, IRMS is also used in poisoning investigations. Through the use of GC/C/IRMS, researchers in Japan analyzed $\delta^{13}$C values in different samples of methamidophos (O, S-dimethyl phosphoramidothioate). High concentrations of this pesticide were identified in some frozen dumplings imported from China, which caused food poisoning in many Japanese citizens who consumed the product [264]. The comparison of Chinese and Japanese methamidophos isotopic values showed that the pesticide was not added in Japan. Subsequent investigations led a temporary worker in China to admit to the crime. Combining isotopes to investigate food contaminated with pesticides for criminal or suicidal reasons has been reported by others. For example, Ehtesham et al. described the use of SIA in a case study of a deliberate contamination of milk powder with a pesticide (MFA) [265]. In another case, the comparison of $\delta^{15}$N and $\delta^{13}$C values of different methyemol (an insecticide) products and a methyemol-containing Soju collected from a fatal
poisoning incident in Korea was one of the investigative leads that enabled detectives to find the poison source and the suspect responsible for the crime [266]. δ15N and δ18O values were also used to show that a combination of horse manure, urine nitrification and intensive evaporation caused an accumulation of nitrates in a water-filled hole that eventually killed 71 wild horses [267].

In some situations, preventative steps have been taken to establish protocols to determine the isotopic ratios of potential poisons. For example, cyanide salts are easily available and highly toxic. Analyses of δ13C and δ15N values in several commercial NaCN and KCN batches, as well as extracted cyanide from food and medicine matrices, support the use of these two isotopes as forensic tools to distinguish different samples [268,269]. Regarding the toxic protein ricin, which is a Schedule I controlled substance from *Ricinus communis* (castor beans), the combination of δ13C, δ15N, δ18O, and δD values provided powerful discrimination between different sources of castor beans even when the preparation method varied between acetone extraction, salt precipitation or affinity chromatography [270]. When a statistical integration approach was taken to join the four isotopes with 87Sr/86Sr ratios, the classification accuracy of castor seeds' geographic origin increased [37]. In other work involving the nerve agent, sarin, Moran et al. has shown that when the precursor methylphosphonic dichloride (DC) is converted to methylphosphonic difluoride (DF) during the synthesis, isotopic fractionation of carbon is minimal [271]. For this reason, nerve agents and their intermediates can be included in, or excluded from, the possibility of deriving from certain precursor feedstocks.

### 10. Questioned documents

Discrimination and source determination of documents remains a severe problem in forensic science. Such examinations include authenticating documents and signatures, document dating and threatening letters, among others [272–276]. Interest in the analysis of paper products was highlighted 2001 during the FBI’s investigations of bioterrorist Anthrax letters in 2001 [277]. The likeness of envelopes used to mail the anthrax spores was investigated through SIA [43], but little discrimination was reported in that case.

Regarding paper examinations, in 2013, Jones and coworkers published a series of three manuscripts depicting the effectiveness of δ13C analysis to distinguish the manufacturing source of document papers [278–280]. Using different brands of office papers collected in Australia and New Zealand, the authors evaluated intra and inter-variability of paper reams and suggested a confidence interval to discriminate documents. More recently, the same group has published two additional manuscripts incorporating oxygen isotope values as well [281,282]. Their results once again showed the potential of IRMS to discriminate paper samples, and a dual-isotope approach using carbon and oxygen was indicated as the next investigation step to optimize and ultimately validate an operation protocol suitable for paper document casework analysis. The results were consistent with previous IRMS results in which carbon, oxygen and hydrogen isotopes were able to discriminate 21 out of 25 European paper documents [283]. The combination of IRMS and XRF appeared to be even more effective at distinguishing between paper products. XRF instruments are more affordable and more-readily accessible to forensic labs and could offer an alternative approach to source attribution.

Regarding ink and toner analysis, Raman spectroscopy and Fourier-Transform Infrared Spectroscopy (FTIR) are the most frequent, reliable and non-destructive methods to differentiate variations found in ballpoint and pens [284–286]. IRMS has only just begun to be explored for inks. For example, Chessen et al. described the first application of SIA of N, C, H, and O to characterize ballpoint and gel inks from pens purchased in the same package, pens from the same brand purchased in three different states, pens of different ages and ink on paper [287]. According to their results, within-package pens have statistically indistinguishable isotopic signatures, but between-package signatures of the same brand pens from different locations and ages were significantly different. In addition, when investigating ink on paper, nitrogen isotope values allowed the discrimination of the different inks. Additional method development is still required before IRMS can be used in questioned-document casework.

### 11. Miscellaneous

The implementation of IRMS in trace evidence studies involving clothing fibers is relatively understudied. Given the fact that alternative analytical methods struggle to provide information other than the polymer type and color of fibers, isotope ratio analysis appears to be a prospective method to determine the source of similar fibers. δ13C, δ15N, and δ18O values have enabled the discrimination between valuable natural fibers of cashmere and cheaper synthetic fibers that were used as a substitute for cashmere in the manufacturing process [288]. Using IRMS, a pilot multi-element study of carbon, hydrogen and oxygen isotopes also showed differences in un-dyed spun cotton fabric fibers from different countries [289]. The authors pointed out that for δD and δ18O values, a larger number of cotton fiber samples would be required to achieve more robust statistical results of within-region variability. To overcome this issue, a subsequent manuscript used hierarchical cluster analysis to compare the isotopic compositions of 17 raw cotton samples from many US states to 15 cotton fibers from different overseas regions [290]. Despite the promising results, some misidentified samples reaffirmed the necessity of a larger dataset and consideration of additional variables that influence results when IRMS is used. The δ13C and δ18O values of cotton can also be used to identify counterfeit currency, since cotton is a primary constituent of paper money. Cerling et al. [8] recently summarized results on how cotton isoscape patterns provide unique geographic information to allow discrimination between genuine US bank notes vs. counterfeit notes.

In recent work by Nienaber et al., SIA showed that within-batch isotope ratio values of cable ties were not significantly different, but that between-batch cable ties were significantly different, especially for δD and δ15N values of the nylon cable ties [41]. Along with IRMS measurements, other physical and chemical measurements enabled the discrimination of 19 of the 20 samples. The only two samples that could not be distinguished were unintentional replicates that shared the same lot number and supplier. Jones et al. surveyed the δD and δ13C values of 26 cling wraps and 26 packages of resealable bags in Australia [291]. Their work demonstrated a significant difference between the within-sample variance and between-sample variance, the benefit of which was extolled in an example case study.

Plastic bags, cling film and adhesive packaging tapes are examples of other materials often associated with forensic situations. Examples include the packaging of drugs and explosives. SIA of plastic wraps have been demonstrated to contribute to the source elucidation of the illicit materials [292–296].

### 12. Summary

This review summarizes some of the SIA applications in different fields of forensic science. Good practices are readily available through the FIRMS Network, and they recommend reliable methods for calibration, normalization and the assessment and reporting of measurement uncertainties. In short, best practices require at least two isotopic standards per element and the reporting of confidence intervals instead of standard deviations.

Ecology and wildlife applications are the most challenging areas of interpretation, and species-specific techniques are often necessary to reveal trophic-level and food-web interactions. There are also big gaps in our understanding of how to interpret IRMS data involving migration patterns and foraging behavior of highly mobile animals. In biological applications, the dynamic nature of different organisms and the differences in metabolism within different organs of an organism are
complicating factors in the interpretation of IRMS results. Special attention should be given to the type of tissues analyzed and their respective turnover rates [4], particularly if focusing in molecules such as amino acids. These are some of the reasons why amino acid isotope analysis became an active and promising research area in eco-geochemistry [297]. The continuous expansion of isotopes metadata and incorporation of other isotope ratios (e.g. $^{87}$Sr/$^{86}$Sr) show potential to leverage the power of interpretations in multiple forensic areas. We expect this increase in data density to be reflected in future datasets, which, combined with advances in chemometrics, should provide a more solid structure for SIA in future casework.

Magnetic sector IRMS instruments still offer better abundance precision than new high resolution mass spectrometers like the Orbitrap [298], but analytical approaches that include techniques such as XRF, Raman spectroscopy, FTIR, NMR, ICP-MS, cavity ring-down spectroscopy, genetic markers, trace elements and fatty acids profiles tend to provide superior discrimination than IRMS values alone. The benefits derive from the inclusion of additional independent variables. The use of chemometrics and statistics in the data analysis has certainly flourished, but still requires further development.

CSIA is recognized as a more sophisticated, and more complex, approach than bulk IRMS. It provides more variables for discrimination, but these variables oftentimes are not true independent variables but covariates, which means a more careful consideration must be taken when interpreting the results. CSIA results provide additional source-level or metabolic information from the samples in question, which is potentially helpful in distinct forensic contexts [61,95]. Baczynski et al. recently reported the development of a modified GC combustion interface that enables a two-order-of-magnitude reduction in the limits of detection [299]. The CSIA measurement of picomolar levels of organics has also been introduced recently [300]. Continued development of PSIA and its applications is likely to provide more in-depth information about the source and metabolism of different amino acids and other metabolites [301]. The value of PSIA to forensic science is questionable at this point, but unless fundamental research continues to push the boundaries of what is possible, we will never move beyond what is currently practicable.

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