Mass Spectrometry in the Diagnosis of Thyroid Disease and in the Study of Thyroid Hormone Metabolism

Marco Borsò[1], Patrizia Agretti[2], Riccardo Zucchi[1], Alessandro Saba[1,3,*]

1. Department of Surgical, Medical and Molecular Pathology and Critical Care Medicine, University of Pisa, Pisa, Italy.
2. Laboratory of Chemistry and Endocrinology, University Hospital of Pisa, Pisa, Italy.
3. Laboratory of Clinical Pathology, University Hospital of Pisa, Pisa, Italy.

*Corresponding author: Alessandro Saba
alessandro.saba@unipi.it
Ospedale S. Chiara, Mass Spectrometry Facility, Building 13
Via Roma 67, 56126 Pisa - Italy
ORCID: 0000-0003-0611-5549

ABSTRACT

The importance of thyroid hormones in the regulation of development, growth and energy metabolism is well known. Over the last decades, mass spectrometry has been extensively used to investigate thyroid hormone metabolism and to discover and characterize new molecules involved in thyroid hormones production, such as Thyrotropin Releasing Hormone. In the earlier period, the quantification methods, usually based on GC-MS, were complicated and time consuming. They were mainly focused on basic research, and were not suitable for clinical diagnostics on a routine basis. The development of the modern mass spectrometers, mainly coupled to liquid chromatography, enabled simpler sample preparation procedures, and the accurate quantification of thyroid hormones, of their precursors, and of their metabolites in biological fluids, tissues, and cells became feasible. Nowadays, molecules of physiological and pathological interest can be assayed also for diagnostic purposes on a routine basis, and mass spectrometry is slowly entering the clinical laboratory.

This review takes stock of the advancements in the field of thyroid metabolism that were carried out with mass spectrometry, with special focus on the use of this technique for the quantification of molecules involved in thyroid diseases.

48 **SUMMARY**

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I. INTRODUCTION

Thyroid hormones (TH), namely triiodothyronine (3,5,3'-triiodothyronine, T_3) and Thyroxine (3,5,3',5'-tetraiodothyronine, T_4), regulate development, energy metabolism, and growth, and their blood levels are controlled by complex central and peripheral signals mainly mediated by hypothalamic Thyrotropin Releasing Hormone (TRH) and pituitary Thyrotropin (Thyroid Stimulating Hormone, TSH). The hypotalamic-pituitary-thyroid axis determines the set point of TH production, and is a highly sensitive negative feedback system in which TH exert a negative regulation of TSH and TRH synthesis and release. In particular, hypothalamic TRH stimulates the synthesis and secretion of pituitary TSH, which acts on the thyroid gland to stimulate all steps of T_3 and T_4 biosynthesis and secretion. Low circulating T_3 and T_4 levels result in increased TRH and TSH production, whereas the opposite occurs when circulating TH are in excess [Melmed et al., 2020]. A diagram of hypotalamic-pituitary-thyroid system is shown in figure 1.

A complete definition of thyroid status requires an accurate measurement of the serum concentrations of T_3, T_4, and TSH to make laboratory tests integral in the diagnosis and management of most thyroid disorders [Larsen, 1982]. Serum determination of TRH, Thyroglobulin, and thyroid hormone metabolites, such as reverse-T_3 (3,3',5'-triiodothyronine, rT_3), 3-iodothyronamine (T_1AM), 3,5-diiodothyronine (3,5-T_2) and 3,3'-diiodothyronine (3,3'-T_2), or thyroid hormone precursors, might be useful to define some pathological conditions and/or for clinical research (figure 2).

Currently, most thyroid biochemical parameters are determined with immunometric methods on high processivity automated instruments. Since the description of the first radioimmunoassay to measure peptide hormones [Yalow & Berson, 1960] and the non-immunogenic steroid hormones [Abraham, 1969], the use of antibodies to measure the concentration of protein and small molecules in clinical samples changed the face of medicine [Hoofnagle & Wener, 2009]. The platform for immunoassays has evolved from the initial competitive radioimmunoassay, to enzyme-linked immunosorbent assay on plastic surfaces, to sandwich liquid-phase chemiluminescent immunometric assay with paramagnetic beads on automated instruments [Bock, 2000], to microfluidic point of care testing "lab on chip" immunoassay [Kartalov et al., 2008]. Despite the efforts made to optimize antibodies and reagents, immunoassays still exhibit several limitations, namely a lack of concordance among platforms, the presence of autoantibodies and/or non-specific heterophilic antibodies, and the high-dose hook effect [Abraham et al., 1969]. Luckily, in most cases thyroid function tests provide a reliable and straightforward picture of the thyroid status. However, in a small but

significant subgroup of subjects, the results of thyroid function tests are confusing due to internal inconsistency or discordance with the clinical picture. Any disagreement between clinical and laboratory data deserves careful attention in order to avoid erroneous diagnoses and treatments, and physicians should closely collaborate with laboratory specialists to interpret hormone assay data. It is always important to carefully check the clinical context by considering confounding factors such as pregnancy, non-thyroid diseases, drug, or supplement therapy. In the absence of these facts, possible laboratory interferences in thyroid function assays should be considered.

Although immunoassays remain the most commonly used method to evaluate hormonal disorders, novel approaches that use liquid chromatography coupled to mass spectrometry detection might solve many of the flaws inherent to immunoassays. Moreover, this technique allows simultaneous measurement of TH and TH metabolites in a biological sample even if present at extremely low concentrations [(Hoefig, Zucchi, & Köhrle, 2016); (Hansen et al., 2016)].

II. THYROTROPIN RELEASING HORMONE

Thyrotropin releasing hormone (TRH), originally named thyrotropin releasing factor (TRF), is a protein that causes different thyroidal and extra-thyroidal effects, among which the feedback regulation of TH secretion is probably the best known. TRH was isolated and characterized in 1969 as a tripeptide pGlu-His-Pro-NH, namely pyroglutamyl-histidyl-proline amide [(Boler et. al., 1969); (Burgus et al., 1969 A); (Burgus et al., 1969 B); (Burgus et al., 1970 A); (Burgus et al., 1970 B)]. This event represented one of the landmark scientific accomplishments of the 20th Century, so that in 1977 to Roger Guillemin and Andrew Schally was granted the Nobel Prize for their discoveries concerning the role of the brain to regulate peripheral endocrine function through the control of the synthesis and section of pituitary hormones [Jackson, 1982].

The TRH plasma level in healthy human subjects ranges between 0.07 and 0.38 nM, with a mean of 0.22 nM, and is not correlated with the thyroid status [(Fröhlich & Wahl, 2019); (Mallik, Wilber, & Pegues, 1982)] because no significant deviation from the normal range is observed in hyperthyroid, hypothyroid, and hypophysectomised subjects [Duntas et al., 1991]. For this reason, the assay of TRH in human serum is of little clinical use. Evaluation of serum TRH level might be useful to indicate the presence of TRH-secreting tumours because high serum levels of TRH-like peptide pyroglutamyl-glutamyl-prolineamide were observed in patients with carcinoid tumors; these data suggest that TRH might be regarded as a cancer biomarker [Klootwijk et al., 1996].

In the early seventies, tests for TRH determination were developed. That improved the investigation of the hypothalamic-pituitary-thyroid axis [(Bassiri & Utiger, 1972 A); (Bassiri & Utiger, 1972 B); (Oliver et al., 1974)]. However, significant difficulties were caused by the presence of specific serum enzymes that inactivate TRH immunoreactivity [(Bassiri & Utiger, 1972 B); (Oliver et al., 1974)]. In particular, TRH incubated in normal human serum or heparinized plasma lost 100% of its immunoreactivity that was recovered after addition of chelating agents such as British-anti Lewisite or 8-hydroxy-quinoline sulfate [May & Donabedian, 1973]. Hence, about a decade later, specific radioimmunoassays to quantify TRH in human serum, which involved a preliminary extraction with methanol precipitation and evaporation, were developed [(Mallik, Wilber, & Pegues, 1982); (Guignier et al., 1981); (Busby et al., 1981 A); (Busby et al., 1981 B)]. In 1991 Duntas et al. described the clinical application of a radioimmunoassay combined with fast protein liquid chromatography to demonstrate that TRH cannot be measured in unextracted blood samples and that TRH levels are not closely related to the thyroid status [Duntas et al., 1991]. These findings suggested that circulating TRH can be derived from extrahypothalamic tissues, predominantly the pancreas.
Interestingly TRH was one of the first natural brain peptides whose amino acid sequence was elucidated with mass spectrometry by Dominic Desiderio, of Horning's group at Baylor Medical School, in collaboration with Guillemin's group. He modified the design of a probe for the direct introduction of methyl- or trifluoroacetyl derivatives of ovine TRH and its putative synthetic peptide into a low resolution LKB 9000 (LKB-Produkter A.B., Stockholm, Sweden) mass spectrometer. Comparison of the acquired spectra showed that ovine TRH was essentially identical to the synthetic peptide, and thus elucidated the amino acid sequence of pGlu-His-Pro-NH for TRH (figure 3) [(Burgus et al, 1970 B); (Desiderio et al., 1971)]. It was demonstrated that TRH biological action is not species specific [Lindsten, 1992].

In contrast to the amazing work carried out with mass spectrometry on TRH characterization, very little was done on TRH quantification in serum or in other biological fluids. In 1977, Heki *et al.* published a Japanese-language paper that described a method based on GC-MS to estimate TRH as a methylated derivative in serum and urine [Heki, Noto, & Hosojima, 1977]. Quantification was based on the intensity of the ion at *m/z* 149, which was the base peak in the EI (electron ionization) spectrum, and corresponded to the fragment methyl-His. The authors reported serum concentrations of 1.55 nM, 0.73 nM, and 1.77 nM in single samples respectively from simple goiter, euthyroid, and hypothyroid subjects, and urinary concentrations of 2.76 nM, 1.66 nM, and 3.59 nM in single samples from hyperthyroid, simple goiter, and hypothyroid subjects, respectively. Actually, serum TRH concentration in

the euthyroid subject was not far from the mean value (0.22 nM) measured in plasma samples with radioimmunoassay [Mallik, Wilber, & Pegues, 1982].

To the best of our knowledge, the only report that dealt with the quantification of TRH in complex biological matrices with LC-MS was published by Chambery *et al.* [Chambery et al., 2010]. The proposed method was primarily based on a single quadrupole mass spectrometer and made use of positive electrospray ionization (ESI) and selected ion monitoring (SIM) of the $[THR+H]^+$ ion, at *m/z* 363.2, to quantify TRH in peptide extracts of normal rat hypothalamus. TRH averaged 0.22±0.02 pmol/mg (calculated from four independent experiments on two rats). The specificity of the method was checked by comparing these results with those obtained with an ion trap mass spectrometer in the selected reaction monitoring (SRM) mode, whose average value was 0.30±0.07 pmol/mg. This result is in a good agreement with those obtained in the SIM mode, as well as with the value of 0.3 pmol/mg tissue measured with radioimmunoassay, reported in 1974 by Winokur and Utiger [Winokur & Utiger, 1974].

III. THYROID STIMULATING HORMONE

Thyroid Stimulating Hormone (TSH), also known as Thyrotropin, is a heterodimeric 28-kDa-glycoprotein hormone secreted by the anterior pituitary gland, under hypothalamic TRH stimulation, that regulates thyroid function. It consists of two peptide subunits, held together by strong noncovalent bonds and co-translationally glycosylated with mannose-rich oligosaccharides (carbohydrates contribute to about 16% of the overall TSH weight): an α subunit, almost identical and highly conserved among different hormones within a single species (luteinizing hormone, LH; follicle-stimulating hormone, FSH; placental hormone chorionic gonadotropin, CG), and a β subunit, specific for TSH that confers immunologic and biologic specificity [Estrada et al., 2014]. Glycosylation occurs at two sites on the α subunits and at a single site on the β subunits. Human TSH carbohydrate chains are subject to variations within the same subject, either in physiological conditions, for instance during the nocturnal TSH surge, or in thyroidal or nonthyroidal disease. TH also modulate TSH synthesis, by decreasing the production rate of both subunits and by regulating the further modifications of the carbohydrate side-chains, together with TRH [Canadian Society of Clinical Chemists, 1992].

The different glycoforms affect TSH bioactivity (i.e., they activate the TSH receptors located on the surface of follicular thyroid cells), cellular iodide uptake, thyroglobulin synthesis, and T_3/T_4 secretion into the blood stream. Thus, the central role that TSH plays in thyroid metabolism makes it the principal diagnostic biomarker of systemic thyroid status.

However, the above- mentioned variability in the glycosylated chains leads to chemical structures that change over time. For this reason, TSH is usually measured on the basis of its biological activity, referred to standard preparations provided by the World Health Organization (WHO), rather than of its concentration. Nowadays, third-generation immunometric methods that possess a functional sensitivity ≤0.01 mIU/L, and work on highly automated instruments, represent the standard of care [(Spencer et al., 1990); (Thienpont et al., 2014); (Owen et al., 2011)].

In this frame, the use of mass spectrometry for TSH quantification does not seem appropriate. On the contrary, mass spectrometry could be the technique of choice for structural investigations, which can be very helpful to set up immunometric methods, as demonstrated by Donadio *et al.*, who compared pituitary TSH and a recombinant form of the same hormone, in order to understand how changes in glycosylation might alter TSH immunoreactivity [Donadio et al., 2005]. This investigation was carried out with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry on samples prepared with sinapinic acid as a matrix. The MS spectrum of the free subunits of highly purified pituitary TSH confirmed that the two N-glycans in the α subunit make it highly heterogeneous and badly resolved, whereas the single glycan β subunit was resolved as five main species that ranged from 14.4 to 15.3 kDa in size (figure 4A). In contrast, the MS spectrum of the recombinant product exhibited subunits of increased molecular weight (figure 4B). Considering that both TSHs share the same peptide sequence, such an increment in weight was due to a significant change in glycosylation. The molecular masses of the TSH glycoforms can also be elucidated with ESI-MS coupled to reversed phase chromatography (RP), as demonstrated by Roepstorff's group in 1995 [Feistner et al., 1995]. This technique was also able to resolve the molecular masses for some glycoforms of β-TSH, but not for those of the more complex α subunits. The deconvoluted molecular masses of the spectra relative to two chromatographic peaks, both attributable to the β subunit, were 14,557, 14,660, 14,727 and 14,830 g/mol for peak 1 (figure 5A) and 14,542, 14,643, 14,712 and 14,815 g/mol for peak 2 (figure 5B). These masses are partly related to each other through a mass difference of 170 u, which can be attributed to a mixture of terminally sialylated and sulfated carbohydrate chains. The difference of 16±1 u between the masses under the two peaks suggests partial oxidation of methionine residues.

IV. SERUM THYROID HORMONES

T_3 and T_4 are essential for growth, differentiation, and metabolism. Most biological effects are due to T_3, because of its greater potency in comparison with T_4 [Chopra, Solomon, & Beall. 1971].

In clinical chemistry, TH are usually assayed in serum and occasionally in plasma. Their quantification in different matrices, such as cerebrospinal fluid, urine, and tissues, provides useful information for the understanding of thyroid metabolism; these findings could reach clinical relevance in the near future.

In serum, the majority of circulating TH are bound to serum carrier proteins, mostly thyroxine-binding-globulin (TBG), transthyretin, and albumin. Blood contains also a very small fraction (about 0.01-0.02%) of T_3 and T_4 as free hormones, which are sometimes regarded as the "biologically active" species, because they are directly accessible to peripheral tissues [(Robbins & Johnson, 1979); (Bartalena & Robbins, 1993); (Schussler, 2000); (Welsh & Soldin, 2016)].

In the 50s, only one test was available to assess the thyroid status. It consisted of an indirect serum determination of total T_4 that used the protein-bound-iodine technique [Benotti & Benotti, 1963]. Between the late 60s and the early 70s, radioimmunoassays able to quantify total T_3 and T_4 in serum or plasma were developed [(Chopra, Solomon, & Beall, 1971); (Chopra, 1972); (Brown et al., 1970); (Larsen, 1972); (Gharib, Mayberry, & Ryan, 1970); (Mitsuma et al., 1972); (Marsden et al., 1975)]. It is obvious that serum total TH (protein-bound plus free hormone) are considerably easier to measure than the free hormones. The measurement of total T_3 and T_4 gives a reliable index of clinical thyroid status in the absence of protein-binding abnormalities. The latter can affect the total T_3 and T_4, and leave the level of unbound hormone unchanged. Increased serum total T_3 and T_4 concentrations might be encountered in euthyroid subjects with TBG excess, familial dysalbuminemic hyperthyroxinemia, and transthyretin-associated hyperthyroxinemia, whereas decreased serum total TH might be associated with TBG deficiency: in these cases, the measurement of serum-free TH levels could be more appropriate for the diagnosis of euthyroidism [Howorth & Maclagan, 1969]. For this reason, many clinicians recommend the assessment of free TH on a routine basis.

To this purpose, different techniques to assay free T_3 (FT_3) and free T_4 (FT_4) were developed with indirect and direct methods. The first approach for indirect estimation of FT_3 and FT_4 was based on the mathematical calculation of the free hormone indexes (FT_3I and FT_4I) from a two-step strategy that involved measurement of the total TH combined with the evaluation of binding protein level. The latter was obtained with direct TBG immunoassay, thyroid hormone-

binding ratio, T_3 resin uptake test, or isotopic determination of the free hormone fraction. Even though these tests often supply inaccurate results, especially in the presence of abnormal levels of binding proteins, they have been extensively used in the clinical practice for more than 40 years [(Faix, 2013); (Midgley, 2001); (Robbins & Rall, 1960)]. Over the years, several immunoassays methods have also been developed to directly measure serum FT_3 and FT_4 concentrations, but quite often they needed extensive sample preparations to separate the free and bound fractions. Currently, highly sensitive and automated immunoassay platforms, that generally use chemiluminescence detection, represent the techniques of choice for the measurement of serum FT_3 and FT_4 in high-throughput clinical laboratories [Bock, 2000]. They are presented as reliable techniques for routine measurements, despite large variations in serum-binding protein concentrations and other factors that can affect immunoassay accuracy. However, they usually include proprietary blockers and binders that make them sensitive to albumin levels, so that their diagnostic accuracy is reduced in case of pregnancy, genetic variations in binding proteins, or treatment with medications that disrupt TH binding to serum proteins [Welsh & Soldin, 2016]. Moreover, many automated immunoassay analyzers label the antigen or the antibody with biotin to take advantage of its very high affinity for streptavidin to decrease non-specific binding [Diamandis & Christopoulos, 1991]. The biotin-streptavidin interaction generates a signal that is quantified and translated into the analyte concentration. As a consequence, a high serum biotin concentration, due to ingestion as a food supplement or in clinical trials (very high biotin dosages have been administered to patients with multiple sclerosis) can interfere with the immunoassay, so that suitable sample preparation becomes necessary [(Bowen et al. 2019); (Kummer, Hermsen, & Distelmaier, 2016); (Trambas et al., 2018)].

Notably, many studies showed inconsistencies between the results of different free thyroid hormone chemiluminescence immunoassay platforms [(d'Herbomez et al., 2003); (Sapin & d'Herbomez, 2003); (Steele et al., 2005); (Giovannini et al., 2011)]. They are probably due to the differential assay susceptibility to alterations in serum binding proteins. Despite the fact that FT_3 and FT_4 immunoassays exhibit wide inter-assay variations, the latest generation assays are very sensitive, with lower limits of quantification (LLOQ) in the order of 0.7 - 0.07 pM that depend on the analytical system used.

Some of the above-mentioned technical limitations of the immunoassays can be overcome with mass spectrometry, which has all the necessary features for accurate measurements of TH and can also be used to detect some of their metabolites, such as iodothyronines and thyronamines.

Actually, mass spectrometry was involved in the quantification of TH, as well as some precursors and metabolites, since the seventies of the past century [Lawson et al., 1974]. At that time, GC-MS was probably the only MS-based technology able to provide a reliable and accurate detection of these molecules. Therefore, many GC-MS methods able to quantify T_3 and T_4 in serum have been reported [(Heki et al., 1976); (Möller, Falk, & Björkhem, 1983); (Ramsden & Farmer, 1984); (Siekmann, 1987); (Thienpont et al., 1994); (Thienpont et al., 1999)]. They usually suffered from laborious and time-consuming sample preparation procedures based on the esterification of the analytes that impacted on recovery and accuracy. Over the last decades, technological implementations of mass spectrometers, in particular the introduction of ESI and atmospheric pressure chemical ionization (APCI) interfaces, enabled an effective coupling with separation techniques in the liquid phase, mainly HPLC and, more recently, UHPLC. The improved selectivity and sensitivity of TH analysis with these novel techniques, coupled to tandem mass spectrometers, led to the first determinations of total T_4 and T_3 in serum described by De Brandabere in 1998 [De Brabandere et al., 1998] and Thienpont in 1999 [Thienpont et al., 1999], who used a HPLC coupled to a triple quadrupole mass spectrometer. The relatively high concentrations of total T_3 and T_4 facilitate their detection and quantification with respect to FT_3 and FT_4, whose concentrations range in the pM. Unfortunately, as already mentioned, many clinicians have a special interest for the free forms [Soldin & Soldin, 2011]; however, this point is still a subject of debate in the clinical community.

LC-MS/MS methods for the quantification of serum TH are considered as the gold standards in clinical chemistry, due to their specificity, sensitivity, accuracy, and precision, which provide a better correlation between TH and TSH with respect to the common immunoassay methods [Welsh & Soldin, 2016]. One of the main strengths of LC-MS/MS is the possibility to use stable isotope-labelled internal standard analogs of the TH of interest. Isotopic dilution methods are widely used in clinical mass spectrometry to monitor the entire process, and to compensate for analytical errors. These internal standards are commercially available for a wide variety of analytes or are in-house synthetized, and their isotopic purity has to be taken into consideration during the development and validation of LC-MS/MS methods.

Another advantage of the MS-based methods is the possibility to translate methods originally developed for the quantification of serum TH, thyroid hormone metabolites (THM), and precursors to different matrices, often with simple modifications to the sample pretreatment procedure to adapt it to the new matrices. On the contrary, the common immunoassay methods

on the market are highly matrix-dependent and require extensive modification to be used with different matrices.

Because hormone concentrations can fluctuate in different physiological and pathological conditions, and reference healthy individuals are difficult to select, the process of bringing analytical and endocrinological demands together will take time [Carvalho, 2012]. An additional technical limitation of LC-MS/MS methods is the low throughput compared to the automated immunoassay platforms.

A. Total Thyroid Hormones

Because TH are largely bound to proteins, a protein precipitation step, followed by TH isolation and purification, is generally required. When GC-MS is used a suitable derivatization process is necessary. Several derivatization methods have been developed, with the aim to make the analytes volatile and ionizable under EI or chemical ionization (CI) conditions. A possible strategy consists in the esterification of the carboxyl functionality and acylation, in particular acetylation, of the amine group [(Möller, Falk, & Björkhem, 1983); (Thienpont, 1994); (Hopley et al., 2004)]; another is the silylation of hydroxyl, carboxyl, and amine functional groups [(Heki et al., 1976); (Heki N, 1978 A); (Heki N, 1978 B)]. Some of these methods were focussed on TH, whereas others allowed the contemporary measurement of some TH precursors (i.e., 3-iodotyrosine (monoiodotyrosine, MIT) and 3,5-diiodotyrosine (diiodotyrosine, DIT)) and metabolites (rT_3, 3,5-T_2, and 3,3'-T_2) [(Heki N, 1978 A); (Heki N, 1978 B)].

Although GC-MS gives an unparalleled chromatographic resolution, LC-MS allows an easier sample preparation and a higher throughput to enable the acquisition of large numbers of samples in a relatively short time. Over the past twenty years, many different LC-MS based methods have been described in the literature. In the method proposed by De Brandabere *et al.* in 1998 and Thienpont *et al.* in 1999, an initial serum protein precipitation step that used acetone was followed by liquid-liquid extraction with ethyl-acetate [(De Brabandere et al., 1998); (Thienpont et al., 1999)]. In the following years, different authors substituted the liquid-liquid extraction with a more-specific and -selective solid-phase extraction (SPE), which could isolate T_3 and T_4, as well as some of their metabolites, from serum matrix and eliminate possible interferences [(Tai, Sniegoski, & Welch, 2002); (Tai et al., 2004); (Van Uytfanghe, Stöckl, & Thienpont, 2004); (Zhang, Conrad, & Conrad, 2005); (Wang & Stapleton, 2010); (Saba et al., 2010)]. In particular, Saba *et al.* described two variants of the same method to detect a recently discovered metabolite of TH, 3-iodothyronamine (T_1AM) (see Section VII),

just like the method proposed in 2008 by Piehl *et al.* [Piehl et al., 2008]. These two method variants shared the sample preparation procedure, but differed for the MS/MS method. The first variant assayed T_3 and T_4, as well as T_1AM, in the SRM positive-ion mode; the other variant quantified T_3, T_4, as well as thyroacetic acid (TA_0) and 3-iodothyroacetic acid (TA_1), in the SRM negative-ion mode, in addition to T_1AM and its deiodinated metabolite thyronamine (T_0AM) in SRM positive ion mode. The instrumental lower limit of detection (LLOD) in the positive-ion mode for T_3 and T_4 was always lower than 1 nM, whereas in the negative-ion mode, it was over double than obtained in the positive ion mode (figure 6). With this method in positive-ion mode, Galli *et al.* [Galli et al., 2012], analyzed 24 samples from patients admitted to a cardiological ward, and 17 from subjects affected by or suspected of thyroid diseases, who were followed up by the cardiovascular risk unit. The results averaged 1.52±0.11 and 142.32±16.20 nM (mean ± SEM) for total T_3 and T_4, respectively. Interestingly, these concentrations were also compared to those of FT_3 and FT_4 measured with chemiluminescent immunoassay, which were 3.18±0.26 and 13.46±0.96 pM, respectively.

The ability of these methods to extract TH from human serum was confirmed in 2004 by Hopely *et al.*, who executed a comparative study of seven different extraction procedures of T_4 from human serum; they found that the combination of protein precipitation and SPE extraction gave more accurate results [Hopley et al., 2004]. In the same year, Soukhova *et al.* proposed a simplified method to extract T_3 and T_4 from human serum, in which the deproteinized samples were subjected to an online extraction prior to the injection into the mass spectrometer [Soukhova, Soldin, & Soldin. 2004]. On-line sample cleanup-based methods, also described by Sakai *et al.* for the measurement of T_3, rT_3, and T_4 [Sakai et al., 2015] could significantly concentrate the samples to increase method sensitivity. As a matter of fact, the LLOQs found by Sakai for all the analytes was about 70 pM.

As a further improvement of these methods, Tai *et al.* proposed the addiction of an antioxidant mixture (i.e., 1,4-dithiothreitol, ascorbic acid, and citric acid) to the samples before the pre-analytical procedure in order to avoid or minimize T_4 to T_3 conversion during sample preparation [Tai et al., 2004].

In 2014, Saba *et al.* modified their previously described methods with the addition of a derivatization step to convert total T_3 and T_4, as well as some of their metabolites and precursors, into the corresponding butyl esters [Saba et al., 2014], according to the procedure proposed by Chace *et al.* [Chace et al., 1993] for amino acids. This method was originally used in cardiac tissues from humans or animals and was optimized also for serum. The derivatization procedure could be carried out either before or after the SPE extraction to yield a ten-fold

increase in sensitivity. The increase resulted from several factors, namely: i) increased ionization efficiency for the esterified TH; ii) increment of molecular weight of T_3 and T_4 by 56 mass units to remove them from some chemical background noise in the SRM mode; iii) modification of matrix composition induced by the very acidic pH used for the esterification reaction. On the other hand, the esterification reaction must be carried out very carefully, in order to avoid undesirable deiodination of T_4 and T_3, promoted by the strongly acidic conditions.

Triple quadrupole mass spectrometer is usually considered as the technology of choice for this kind of analysis in clinical diagnostics because of its proven ruggedness. However, recently Alvarez *et al.* [Álvarez, Madrid, & Marazuela, 2016] proposed the first method for the quantification of total serum TH and THM with a hybrid mass spectrometer, LC-QTOF, to demonstrate that a TOF analyzer can be profitably used to characterize and quantify total serum TH in place of the third quadrupole of the common triple quadrupoles. The high resolution is an added value of this technique. Jongejan *et al.* [Jongejan et al., 2020] also proposed a sensitive high-throughput method for total TH in serum based on the use of the hybrid mass spectrometer Sciex QTRAP 6500+, which basically is a tandem mass spectrometer with a linear ion trap (LIT) that replace the third quadrupole (LC-QLIT). However, this mass spectrometer was used just as a traditional triple quadrupole, by performing all the acquisitions in SRM. In addition to total TH (LLOQs: 44.5 pM for T_3 and 4.1 nM for T_4), this method assessed five iodothyronines: thyronine (T_0), 3-iodothyronine (3-T1), 3,5-T2, 3,3'-T2, and rT3 with electrospray ionization in the positive-ion mode, and two iodothyroacetic acids (3,5,3'-triiodothyroacetic acid (Triac, TA_3) and 3,5,3',5'-tetraiodothyroacetic acid (Tetrac, TA_4) in the negative-ion mode (see Section VII).

In conclusion, different methods and techniques can be employed to quantify total TH in serum. On this basis, several years ago the National Institute of Standards and Technology (NIST) adopted two reference measurement procedures (RMP) in compliance with regulatory requisites. The method proposed by Tai *et al.* [Tai et al., 2004] was chosen for the quantification of total T_3, whereas that proposed by Wang and Stapleton [Wang & Stapleton, 2010] for the quantification of total T_4 [Richards et al., 2017].

Inductively Coupled Plasma (ICP) mass spectrometry-based methods have also been proposed in order to detect elemental iodine for a sensitive quantification of total and free TH in serum [Long et al., 2016]. In 2000, Michalke *et al.* described a RP-HPLC-ICP-MS method based on human serum iodine speciation to measure, beside total TH, also their precursors MIT and DIT, and their metabolite reverse-T_3 [(Michalke, Schramel, & Witte, 2000 A); (Michalke,

Schramel, & Witte, 2000 B)]. A protease treatment was necessary to separate the transport proteins from the protein-linked hormones, because they did not interact with the LC stationary phase, and therefore they were not retained on the column. Without proteolysis, signals attributable to free TH were observed, but their concentrations were too low to be measured. Interestingly, the studies about the speciation of iodine with HPLC-ICP-MS were preceded by an iodine assay method based on capillary electrophoresis (CE) coupled to ICP-MS [Michalke & Schramel, 1999]. This valuable separation technique separates and quantifies TH, as well as iodide and iodate, with detection limits for T_3 and T_4 of 6.6 mM and 9.2 mM, respectively. Despite the small sample amounts usually injected in CE, these values are close to those achieved with HPLC-ICP-MS, which were 2.3 mM and 1.0 mM, respectively.

B. Free Thyroid Hormones

Quantification of the free fractions requires sample pre-treatment prior to the mass spectrometry analysis, in order to remove the protein-bound TH without interfering with labile non-covalent interactions that bind TH to their carrier proteins. Because denaturing solvents cannot be used, the general strategy consists in the physical isolation of FT_3 and FT_4. To this end, equilibrium dialysis and ultrafiltration techniques have been proposed [Yue et al., 2008]. Both procedures show potential limitations. In equilibrium dialysis, a possible drawback is represented by sample dilution and by the potential influence of the buffer on the equilibrium between the free and the bound thyroxines (e.g., it could contain adsorbing components). Conversely, the main disadvantages of ultrafiltration are related to adsorption of the analyte of interest at the membrane, protein leakage, and the need for an optimal control of temperature and pH. These two types of approaches have frequently produced differences in quantitative results and no agreement has been reached on the most-convenient procedure [Holm et al., 2004].

The first equilibrium dialysis method able to quantitate FT_4 in human sera has been proposed by Van Uytfanghe *et al.* [Van Uytfanghe et al., 2006]. In this method, the dialysate was further purified with SPE before the LC-MS/MS analysis. In the following years, equilibrium dialysis procedures coupled to LC-MS/MS were successfully used by several authors [(Van Uytfanghe et al., 2006); (Thienpont, Beastall, & Christofides, 2007); (IFCC et al., 2007); (Yue et al., 2008); (La'ulu, Rasmussen, & Straseski, 2016)], even if these techniques were time consuming, and required expensive devices not available in most clinical laboratories.

In 2005, Soldin *et al.* proposed a novel ultrafiltration method for the isolation and LC-MS/MS quantification of FT_4 that made use of an AB-Sciex (Concord, ON, Canada) API 4000

tandem mass spectrometer, which at that time was a top-level instrument but is nowadays regarded as a medium-sensitivity instrument [Soldin et al., 2005]. The serum-free fraction was extracted with a relatively cheap and disposable ultrafiltration device, the Millipore (Burlington, MA, USA) Centrifree YM-30, and 650 µL of the resulting filtrate were injected into the HPLC-MS/MS device, which included an on-line clean-up preceding the chromatographic separation [Gu, Soldin, & Soldin, 2007]. The contribution of on-line clean-up, which was demonstrated also in the quantification of different classes of compounds such as immunosuppressants [Koal et al., 2004] and steroid hormones [(Saba et al, 2009); (Dovio et al., 2010)], consisted in the possibility to inject large amounts of sample and in the removal of some of the matrix components that induced ion-suppression effects. With this method, studies of clinical interest in subjects with different physiological and pathological states were profitably carried out [(Kahric-Janicic et al., 2007); (Jonklaas et al., 2009); (Soldin & Soldin, 2011)]. In the recent past, technological breakthroughs led to new instruments with an increased sensitivity that allowed an easier and more-reliable quantification of these analytes [(Kiebooms et al., 2014); (Tanoue et al., 2018)].

V. SERUM REVERSE T_3

In 1971, Surks and Oppenheimer demonstrated that T_4 mono-deiodination might occur not only in the phenolic-ring (outer) to produce T_3, but also in the tyrosyl-ring (inner) to produce rT_3 [Surks & Oppenheimer, 1971]. rT_3 has long been regarded as metabolically inactive, and it was considered as a competitive inhibitor of T_3. Its clinical significance is, however, debated and has not been completely clarified. In the clinical setting, rT_3 measurements can be helpful when the assays of serum TSH, FT_4, and FT_3 do not support the diagnosis of suspected thyroid dysfunction and additional information is required, particularly to differentiate between hypothyroidism and non-thyroidal illness. In these cases, the T_3 to rT_3 ratio is a diagnostic tool to investigate pathological alterations of TH metabolism [Kumar et al., 2010].

Although rT_3 is rarely assessed on a routine basis, the method usually used for this purpose is RIA. However, the pressure to limit the use radioactive material has encouraged the shift to LC-MS/MS, which is always superior in specificity with respect to immunoassays. Zhang *et al.* developed the first ESI-MS/MS method to detect and quantify T_3 and rT_3 in human sera [Zhang, Conrad & Conrad, 2005]. The molar percentages of T_3 and rT_3 were 81.5±2.4 and 18.5±2.4, respectively, with a T_3/rT_3 ratio of 4.5±0.7. Smaller ratios were obtained by Jongejan *et al.*, who reported a median ratio of about 3 [Jongejan et al., 2020], although the ratio widely varied under pathological conditions. Sakai *et al.* compared RIA (RIAZEN Reverse T_3,

ZenTech, Angleur, Belgium) and the already mentioned in-house developed HPLC-MS/MS method with on-line SPE [(Sakai et al., 2015); (Sakai et al., 2016)]. They observed a good correlation between the two techniques ($r = 0.928$, $p < 0.001$) at concentrations lower than 1.1 nM. However, the slope of the linear regression equation was 2.48, as a consequence of the significantly lower concentrations usually measured with HPLC-MS/MS. The reason for this discrepancy is not completely clear, but it can be largely attributed to cross-reactivity. In 1974, Chopra reported that 3,3'-diiodothyronine (3,3'-T_2) had 10% cross-reactivity with rT_3-binding sites on the antiserum, whereas thyroxine (T_4) and triiodothyronine (T_3) cross-reacted by less than 0.1% [Chopra, 1974]. However, their contribution to the MS/RIA discrepancy is probably more relevant because serum T_4 concentration is several orders of magnitude higher than 3,3'-T_2 concentration. T_4 cross-reactivity would be particularly significant in hyperthyroid patients. Consistent with this hypothesis, when rT_3 concentrations were in the range 1.1–3 nM, the comparison between RIA and HPLC-MS/MS provided a linear regression equation with a less-steep slope [Mathur et al., 1979]. The lower sensitivity of HPLC-MS/MS (LLOQ 0.077 nM) with respect to RIA (LLOQ 0.014 ng/mL) does not compromise the clinical use of the former method for rT_3 assay [(Sakai et al., 2015); (Sakai et al., 2016)]. A slightly lower LLOQ (0.031 nM) was reported by Jongejan *et al.* who used out a high-end tandem mass spectrometer; *i.e.*, Sciex Sciex QTRAP 6500, preceded by off-line SPE [Jongejan et al., 2020]. In this frame, the results described by Bowerbank *et al.* probably are overly optimistic because their LLOQ were 0.0006 nM with HPLC-MS/MS preceded with off-line SPE, 0.0015 nM with electro-chemiluminescence immunoassay (ECLIA) and 0.0037 nM with enzyme-linked immunosorbent assay (ELISA). The ELISA result is particularly surprising, because it is 4-fold lower than LLOQ obtained with RIA [(Sakai et al., 2015); (Sakai et al., 2016)].

VI. ASSAY OF THYROID HORMONES AND REVERSE T_3 IN MATRICES OTHER THAN SERUM

At present, the clinical interest to assay TH and their metabolites in matrices other than serum, namely urine, cerebrospinal fluid (CSF), tissues, and cells, is limited. However, the functional response to TH depends on the concentration that exists at the receptor level, and several lines of research have demonstrated that peripheral metabolism and tissue uptake are crucial regulatory steps. Thus, current research gives increasing emphasis to the determination of tissue TH levels, as well as to some TH metabolites that appear to produce local and systemic effects.

Only a few immunoassay methods to assess TH in matrices other than serum have been developed. More often, the assay was improperly carried out with methods optimized and validated for serum. These considerations also hold for TH metabolites, for which the few immunometric methods available are affected by critical issues, such as their uncertain selectivity. Therefore, most investigators believe that non-serum assays should rather be based on mass spectrometry. It should also be considered that, when a MS-based method has been optimized for a certain matrix, its conversion to another matrix is often possible, although it usually requires adjustments, particularly to the sample-preparation protocol.

With regard to matrices that are easily available in patients, pilot studies have been performed in urine, saliva, and milk.

Free TH are partly filtered by the renal glomeruli, which act as *in vivo* dialysis devices, and excreted into urine. The renal clearance of the free fractions of TH correlate well with serum-free hormone levels [Cai, 2014]. The main advantage of measuring TH in a 24-hour urine collection sample is that thyroid status is assessed over an extended time frame, in contrast to the serum assay that provides information only at the time of venipuncture. For this reason, urine samples have a significant diagnostic value in the detection of some disease states [Chan, 1974]. However, only a few methods specifically developed for urine are available in the literature, including those based on mass spectrometry. An interesting method was developed by Fan *et al.* to quantify T_4, T_3, and rT_3, with HPLC-ICP-MS preceded by stir-bar sorptive extraction (SBSE), that is a derived from the well-known solid-phase microextraction (SPME) and provides higher extraction efficiency of target analytes [Baltussen et al., 1999]. Unfortunately, Fan *et al.* focused their research on method optimization, and few data about the assessment of the endogenous hormones were provided. However, it is interesting to observe that in urine rT_3 concentration exceeded T_3 concentration [Fan et al., 2013].

The first validated method able to quantify T_4 in saliva samples (pg/mL) was developed in 2011 by Higashi *et al.* [Higashi et al., 2011]. In spite of the small number of patients, they were able to report an increase of T_4 concentration in patients affected by Grave's disease, versus euthyroid subjects. Recently, Li *et al.* used their LC-MS/MS method to quantify levels of TH in human breast milk and reported concentrations in the low nM range [Li et al., 2020].

Cerebrospinal fluid (CSF) is less easily available, but can provide more-interesting clinical information, because of the putative role of local (i.e., cerebral) TH metabolism in some neurological diseases. In 2017, for the first time, Accorroni *et al.* used an LC-MS/MS technique to assay T_4, T_3, and rT_3 in 35 human patients [Accorroni et al., 2017]. CSF concentrations were in the low nM range for T_4, and in the pM range for T_3 and rT_3. Interestingly, in Alzheimer's

disease, but not in fronto-temporal dementia, a significant correlation was observed between a clinical index of cognitive dysfunction and rT_3/T_3 ratio.

Several investigators assayed TH in animal and occasionally also in human tissues, and used a variety of LC-MS/MS methods. Butt *et al.* and Noyes *et al.* applied a previously validated method [Wang & Stepleton, 2010] in human liver microsomes [Butt, Wang, & Stapleton, 2011], and in juvenile fathead minnows (*Pimephales promenas*) [(Noyes, Hinton, & Stapleton, 2011);(Noyes et al., 2013)]. Kunisue *et al.* developed a new method that was used to quantitate T_4, T_3, and rT_3 in rat thyroid gland and brain [(Kunisue et al., 2010); (Kunisue, Fisher, & Kannan, 2011 A); (Kunisue, Fisher, & Kannan, 2011 B)], as well as in zebrafish muscle [Little et al., 2013]. Bussy *et al.* also used LC-MS/MS to quantify TH in sea lamprey (*Petromyzon marinus*) larval tissues [Bussy et al., 2017], whereas Laslo et al. measured T_4, T_3 and rT_3 in pooled *Eleutherodactylus coqui* embryos [Laslo, Denver, & Hanken, 2019]. Ackermans *et al.* validated an UPLC-MS/MS method to identify and quantify TH and their metabolites in various animal tissues [Ackermans et al., 2012]. They analyzed rat liver, heart, hypothalamus, and thyroid homogenate, and detected T_3 and T_4 in all tissues whereas rT_3 was quantifiable only in thyroid. Saba *et al.* also detected T_3 and T_4 in virtually every rat tissue with an LC-MS/MS method [Saba et al., 2010]. The same method was later used by several authors to quantify cardiac TH levels in rat model heart failure [(Pol et al., 2011); (Weltman et al., 2013); (Weltman et al., 2014); (Weltman et al., 2015)].

In general, these investigations provided tissue T_3 and T_4 concentrations on the order of 0.5-50 pmol/g. Whereas these results have provided relevant biological information, it should be stressed that tissue assays have not yet been properly validated and standardized. Quality control data, such as accuracy, precision, recovery, process efficiency, and matrix effects have been appropriately determined only in a few studies [Köhrle, 2020]. It is also unclear what is the more-appropriate pre-analytical procedure. Different alternatives have been proposed for tissue homogenization (as an example, disposable bead beating devices, which can finely grind samples, vs traditional homogenizer, such as Potter Elvejheim), protein precipitation (during or after homogenization, with different solvents and pH conditions) and analyte extraction (liquid/liquid extraction vs solid phase extraction (SPE)). It is likely that the ideal procedure should be targeted to the specific tissue. For instance, SPE might provide a higher signal to noise ratio and ensure better results in liver, heart, or kidney, whereas liquid/liquid extraction allows a higher recovery and might be preferred in lipid-rich tissues, such as brain and adipose tissue [Donzelli et al., 2016].

Specific technical improvements have recently been proposed. In 2014, Saba *et al.* validated a novel HPLC-MS/MS method that included a derivatization step to improve shape and intensity of TH peaks [Saba et al., 2014]. After protein precipitation and SPE, dried residues of serum samples were derivatized with 3.0 N hydrochloric acid in n-butanol to form the corresponding butyl esters of T_3 and T_4. This procedure was associated with increased ionization efficiency of esterified TH and remarkable reduction of background noise, so that satisfactory results were obtained with biopsies weighing about 50 mg (figure 7). This method was used to assay T_3 and T_4 in human left ventricle myocardial biopsies and measured concentrations of 1.51±0.16 and 5.94±0.63 pmol/g, respectively. In an experimental investigation performed in hypothyroid and hyperthyroid rats, this technique revealed a significant mismatch between the changes in TH that occurred in serum and in specific tissues [Donzelli et al., 2016]. The main drawback of the tissue assay was its limited accuracy (70-75%), which might probably be improved with the optimization of the homogenization procedure. A similar derivatization procedure was used by Chen *et al.* to assay T_4, T_3, and rT_3 in zebrafish larvae, after sample digestion with primase and SPE [Chen et al., 2017].

A different methodological improvement was proposed by Ruuskanen *et al.*, who developed the first LC-nano flow- triple quadrupole mass spectrometric method to quantify T_3 and T_4 in the amol range. The validated method was used to quantify TH in egg yolk samples of several species of birds. [Ruuskanen et al., 2018].

Adoption of a method that uses a nano-UPLC system with micro flows interfaced with a quadrupole time-of-flight mass spectrometer was proposed by DeAngelis *et al.* [De Angelis et al., 2016]. This approach decreases the amount of sample necessary for the analysis (about 50-100 mg), which underwent liquid-liquid extraction and SPE. T_3 and T_4 were detectable in virtually in all mouse tissues, whereas rT_3 was always below the limit of quantification (0.75 ng/mL). The same method was applied to measure TH in human and rat placenta [(Li et al., 2018 A); (Li et al., 2018 B)]. The reported concentrations of T_3, T_4, and rT_3 ranged in ng/g, and agreed with a previous study based on LC-MS/MS [Leonetti et al., 2016]. Notably, placental assays have potential clinical importance. During pregnancy, maternal TH are delivered to the fetus through the placenta, and even minor changes in their circulation can affect the normal development of the brain and other organs. The quantification of TH and of some of their metabolites in placenta would provide useful diagnostic and predictive information.

VII. THYROID HORMONE METABOLITES AND PRECURSORS

The classical paradigm maintains that T_4 can be activated in peripheral tissues by outer-ring deiodination to yield T_3 that is regarded as the active hormone, because its affinity for nuclear TH receptors is several orders of magnitude higher than T_4 affinity. Other deiodinations and several additional reactions have been reported, and they were initially considered as inactivation reactions. The responsible enzymes include, beside the three well-known selenoprotein deiodinases [Bianco et al., 2002]: sulfotransferases and glucuronidases, which conjugate the phenolic hydroxyl group of TH; amine oxidases and aminotransferase, which remove the amino group from the side chain, to yield α-chetoacids; decarboxylases, to lead to the production of biogenic amines. These reactions can occur in different combinations so that a very large number of derivatives can be theoretically produced, and most of them have actually been detected in biological systems. Excellent recent reviews on various features of TH metabolism are available, and the reader is referred to them [(Hoefig, Zucchi & Köhrle, 2016); (Rutigliano & Zucchi, 2017); (Zucchi, Rutigliano, & Saponaro, 2019); (Giammanco et al, 2020); (Homuth et al., 2020), (Köhrle, 2020)].

The interest in TH metabolism is increased recently, since it was proposed that, contrary to the classical view, some metabolites may represent additional chemical messengers. In fact, some derivatives preserve a high affinity for nuclear thyroid hormone receptors (e.g., 3,5,3′-triiodothyroacetic acid, also known as Triac, and 3,5,3',5′-tetraiodothyroacetic acid, also known as Tetrac), or interact with other receptors. In particular, 3,5-dioiodothyronine (3,5-T_2) appears to interact with incompletely-identified mitochondrial targets [Senese et al., 2018], and 3-iodothyronamine (T1AM) is a high-affinity ligand of a G-protein coupled receptor known as Trace Amine-Associated Receptor 1 (TAAR1) [(Scanlan et al., 2004); (Rutigliano, Accorroni, & Zucchi, 2018)], although it can also interact with other aminergic G-protein coupled receptors, ionic channel of the transient receptor potential (TRP) family, and possibly additional molecular targets [(Hoefig et al., 2016); (Koehrle & Biebermann, 2019)].

In this frame, the possibility to develop and validate mass spectrometric methods, coupled to either gas phase or liquid phase separation techniques, can be profitably exploited. In recent years, a large number of methods have been described that can assay, beside TH, also TH precursors, mostly MIT and DIT, as well as TH metabolites, particularly those which are regarded as additional chemical messengers. Here, we propose a brief summary of some of them.

675 **A. Diiodothyronines**

676 Since the 80's, the debate on the potential role of the TH metabolite 3,5-T_2 and its main isomer,
677 3,3'-T_2, triggered the analytical challenge for their quantification in serum. Several
678 immunoassays were developed and used in healthy individuals and in subjects affected by
679 thyroid diseases [Chopra, 1996]. Although the detected concentration was method-dependent
680 and usually lied in the nanomolar range, a major methodological concern was the extent of
681 cross-reactivity with T_3. Recently, a competitive chemiluminescence immunoassay (CLIA)
682 based on monoclonal antibodies has been developed by Köhrle's group to yield results in the
683 range of 150-700 pM. However, about one-third of the sample measurements was below the
684 lower limit of quantitation [Lehmphul et al., 2014].

685 ESI-MS/MS can distinguish the T_2 isomers in biological samples [Zhang et al., 2006] and
686 some authors included 3,5-T_2 and 3,3'-T_2 in their LC-MS/MS methods together with T_3, T_4
687 and rT_3 [(Wang & Stepleton, 2010); (Kunisue et al., 2011)] to investigate whether it could be
688 detected in animal and human serum. Soldin and Soldin [Soldin & Soldin, 2015], with their
689 patented method for the simultaneous quantification of TH, rT3, and T1AM, found serum
690 reference concentration intervals (2.5th to 97.5th percentile) for 3,3'-T_2 of 13.7-46.5 and 17.9-
691 58.3 pM for females and males, respectively. These values are in a good agreement with those
692 reported by Jonklass *et al.* (12.8-43.8 pM) [Jonklass et al., 2014], which made use of the same
693 method patented by Soldin and Soldin [Soldin & Soldin, 2015], whereas Jongejan *et al.*
694 obtained lower values (4.76-14.66 pM) [Jongejan et al.,2020]. Conversely, Richards *et al.*
695 achieved higher concentrations (± standard deviation) 79±22 pM [Richards et al., 2019], and
696 even higher values (253±29 pM, mean±SEM) were reported by Lorenzini *et al.* in a limited
697 number of samples from supposedly healthy subjects [Lorenzini et al., 2019]. In the same
698 publication, Lorenzini *et al.* firstly reported serum 3,5-T_2 concentrations assayed with LC-
699 MS/MS, which were on average three-times lower than those of 3,3'-T_2, and ranged 5.37-242.6
700 pM (78±9 pM, mean±SEM). In contrast, Richards *et al.* did not detect any endogenous 3,5-T_2
701 with their HPLC-MS/MS method, whereas Jongejan *et al.* detected it just in a few samples
702 [(Richards et al., 2019); (Jongejan et al., 2020)].

703 It has been discussed whether the higher amount of endogenous 3,5-T_2 reported by Lorenzini
704 *et al.* might be accounted for by contamination of the isotope-labelled internal standard, 3,5-
705 diiodotyronine-$^{13}C_9$-^{15}N ($^{13}C_9$-^{15}N-T_2), with unlabeled 3,5-T_2 [(Richards et al., 2019); (Köhrle
706 et al., 2020)]. However, as a matter of fact the amount of 3,5-T_2 in $^{13}C_9$-^{15}N-T_2 was
707 insignificant, as confirmed by the SRM chromatogram of 3,5-T_2 from a water solution of the
708 internal standard at the same concentration used for the human serum samples, namely 1.9 nM

(figure 8). On the other hand, the pre-analytical sample processing developed by Lorenzini *et al.* was more complex, but probably more efficient, than the procedure used by Richards *et al.*. Lorenzini *et al.* performed the SPE extraction, which is a very critical step, with single cartridges that allow percolation of eluents, and thus provide a slow and efficient extraction. Lipid removal with suitable liquid extraction was also carefully optimized and proved to be effective. Furthermore, a relatively large amount of starting material (human serum) was used and concentrated up to 40-fold. On the contrary, Richards *et al.* used a simpler extraction procedure based on SPE well plates, where solvent elution was carried out under vacuum, which could reduce analyte recovery because TH and their metabolites are usually not tightly retained onto medium cation exchange (MCX) stationary phase. They also carried out lipid removal with well plates and, although the Ostro stationary phases are well reputed, their clean-up efficiency could be lower than allowed by optimized liquid extraction. Moreover, they did not concentrate the sample and, although they used a top-level mass spectrometer, this decision might have been disadvantageous.

In conclusion, the quantification of 3,3'-T_2 is not really critical, because its biological significance is still unclear and, therefore, the interest of the clinical community in this metabolite is limited. In contrast, 3,5-T_2 assay might have clinical relevance and the analytical methods based on mass spectrometry are promising, although further developments aimed to increase sensitivity and reduce its technical complexity are still necessary to make this tool amenable to large-scale clinical use.

B. 3-Iodothyronamine and its metabolites

The discovery of T_1AM, an endogenous derivative of TH, spurred researchers to investigate this novel metabolite and related compounds. In 2004, its presence was firstly reported in rat brains with a LC-MS/MS method developed by Scanlan *et al.* [Scanlan et al., 2004]. The endogenous biosynthesis of T_1AM involves a series of deiodination and decarboxylation of thyroidal or peripheral TH precursors. Further metabolism ensues from different types of reactions, namely: oxidative deamination to TA_1; deiodination to yield T_0AM; N-acetylation to form N-Ac-T_1AM; esterification to provide the corresponding glucuronide (T_1AM-glucuronide) and sulfonate (O-sulfonate-T_1AM) derivatives. The precise metabolic pathways responsible for T_1AM biosynthesis and metabolism are still incompletely clarified, and they are extensively discussed in some recent reviews [(Köhrle & Biebermann, 2019); (Hoefig, Zucchi, & Köhrle, 2016)]. In any case, the administration of exogenous T_1AM to experimental animals elicited a variety of functional effects, and endogenous T_1AM is likely to play a

743 significant role in the regulation of neural functions and/or energy metabolism [(Köhrle &
744 Biebermann, 2019); (Hoefig, Zucchi, & Köhrle, 2016); (Zucchi, Accorroni, & Chiellini, 2014);
745 (Köhrle, 2019)]. Therefore, there is a strong interest to develop methods to detect and quantify
746 T_1AM in biological matrices, and in this regard LC-MS/MS is considered as the gold standard
747 technique.
748 Despite the fact that numerous authors designed accurate and sensitive LC-MS/MS methods
749 to detect T_1AM in serum, the effective concentration of endogenous T_1AM (and also its
750 metabolite TA_1) in serum is still debated. Difficulties to measure these analytes in blood,
751 caused by binding to serum carrier protein ApoB100 and low endogenous concentration (pM),
752 required extensive pre-analytical sample preparation and high instrumental sensitivity.
753 Several sample preparation methods, often based on different technologies, are reported in
754 the literature [(Braulke et al., 2008); (DeBarber et al., 2008); (Saba et al., 2010); (Ackermans
755 et al., 2010); (Galli et al., 2014); (Soldin & Soldin, 2015); (Richards et al., 2019)]. In particular,
756 the sample preparation from Ackermans *et al.* consisted of the incubation with proteinase K to
757 degrade the carrier proteins, among which ApoB100, and the on-line SPE that used a mixed-
758 mode weak cation exchange column to extract the analytes from the matrix and remove the
759 proteinase K debris, which might cause ion-suppression [Ackermans et al., 2010]. The
760 efficiency of on-line clean-up was confirmed by the good method sensitivity that provided a
761 LLOD for T_1AM of 0.08 nM, despite a moderate instrumental sensitivity, and by the significant
762 advantages in terms of throughput. Hansen *et al.* used a more conventional off-line SPE for the
763 extraction of T_1AM, as well as Tetrac, Triac, Diac (3,5-diiodothyroacetic acid), and various
764 iodothyronines [Hansen et al., 2016]. The SPE extraction probably was mainly optimized for
765 iodothyronines and thyroacetic acids, because the mixed-mode strong anion exchange material
766 used is not the best choice for thyronamines, which are poorly retained by anion exchange and
767 interact mostly with the sorbent's retentive component. However, the good instrumental
768 sensitivity contributed to a LLOD for T_1AM of 0.1 nM. Richards *et al.* also used mixed-mode
769 SPE sorbent materials; in this case contained in 96 well plates, but with a medium cation
770 exchange component [Richards et al., 2019]. As mentioned in the previous section, the SPE
771 was carried out under vacuum, which might affect the physical interactions between analyte
772 and stationary phase to reduce cleaning efficiency and recovery. It was confirmed with a LLOD
773 for T_1AM of only 0.1 nM, although the authors used the highly sensitive Sciex 6500 QTrap
774 mass spectrometer and, respectively, two- and four-times the sample volume used by
775 Ackermans *et al.* and Hansen *et al.* [(Ackermans et al., 2010); (Hansen et al., 2016)]. The
776 method set up by Saba *et al.* was based on a complicated and time consuming sample

preparation procedure that limited the loss of T_1AM and of the other analytes during protein precipitation. Moreover, the off-line SPE, which was carried out at atmospheric pressure (no vacuum was applied) under optimized pH conditions, provided high SPE recoveries and low matrix effects. This procedure provided a general high sensitivity, and lowered the limit of detection of T_1AM to 35 pM, and those of T_3 and T_4 to 14 and 11 pM (vs 25 pM and 50 pM obtained by Richards *et al.*), respectively [(Saba et al., 2010); (Galli et al., 2014); (Lorenzini et al., 2019)]. As a matter of fact, the methods from Saba *et al.* was one of the few methods that detected endogenous T_1AM in human serum; the reported concentration was 0.219±0.012 nM (mean±SEM). These values are quite different from those obtained by a validated chemiluminescence immunoassay method (CLIA) based on mouse monoclonal T_1AM antibodies, which provided median serum concentrations of 66±26 nM [Hoefig et al., 2011]. This technique has been used to assay T_1AM in different conditions, and variations of endogenous T_1AM concentration have been reported in heart failure [la Cour et al., 2019].

At present, no definite explanation for the divergent results obtained with CLIA vs LC-MS/MS has been obtained. It has been speculated that the mass spectrometry-based method might detect the free T_1AM fraction, which is expected to be <1% of total T_1AM, putatively detected with CLIA. However, there is no direct evidence to support this hypothesis, and the experience with TH shows that, unless specific physical separation methods are used, mass spectrometry-based methods yield total rather than free T_3 and T_4, as discussed above. The fact that CLIA yielded higher concentrations might be alternatively due to cross-reactivity of the antibody with endogenous interferents, although Hoefig *et al.* excluded significant affinity for many different iodothyronines and iodothyronamines [Hoefig et al., 2011]. Interestingly, Lorenzini *et al.* obtained evidence that serum amine oxidase might oxidize T_1AM and favor the formation of protein adducts (e.g. Schiff bases) [Lorenzini et al., 2017]. Because the antibody used by Hoefig *et al.* was actually raised vs T_1AM linked to albumin, it is possible that the CLIA technique also detects such adducts. Further investigations will be necessary to clarify this issue and to evaluate the existence and potential functional effects, if any, of T_1AM adducts.

Apart from the serum assay, the LC-MS/MS technique played a fundamental role to better understand the effects of T_1AM and its metabolites in animal experiments and in different types of tissues and cell lines [(Scanlan et al., 2004); (Chiellini et al., 2007); (Agretti et al., 2011); (Orsi et al., 2011); (Manni et al., 2012); (Manni et al., 2013); (Musilli et al., 2014); (Ghelardoni et al., 2014); (Mariotti et al., 2014); (Orsi et al., 2014); (Laurino et al., 2015); (Hansen et al., 2016); (Assadi-Porter et al., 2018); (Lehmphul, Hoefig, & Köhrle, 2018); (Accorroni et al.,

2020)]. A large part of these experiments made use of the LC-MS/MS method set up by Saba *et al.* [Saba et al., 2010] and its following developments, with several adjustments to the sample-preparation procedure in order to make it compatible with the different matrices (figure 9). As an example, we report here a possible sample-preparation procedure to be used for liver and other tissues. It consists in the following steps: tissue homogenization, achieved by placing the sample in disposable vials with ceramic beads together with 1 ml of phosphate-buffered saline (PBS); incubation at 37°C with a solution of the internal standards; deproteinization with 1 ml of ice cold acetonitrile; centrifugation; washing of the supernatant (3 times) with 2 ml hexane each time; drying under nitrogen at 40°C; and reconstitution with a 70/30 (V/V) water:methanol mixture prior the HPLC-MS/MS analysis. This procedure is quite simple end efficient, but usually requires > 150 mg/sample [Lorenzini et al., 2017]. When smaller amounts of tissue are available, or strong ionic suppression effects induced by the matrices are present, sample clean-up could be improved with the extraction of the homogenized sample supernatant with the SPE-based procedure also used for TH and T_1AM in serum [Saba et al., 2010], followed by the Fischer esterification of the dried eluate with 3.0 N hydrochloric acid in n-butanol prior the HPLC-MS/MS quantification [Saba et al., 2014]. For a summary of the endogenous levels of T_1AM detected in different tissues the reader is referred to specific reviews [(Hoefig et al., 2016); (Koehrle & Biebermann, 2019)].

Interestingly, Zhang *et al.* used MALDI-MS imaging to detect T_1AM in mouse brain slices 30 and 60 min after intraperitoneal administration [Zhang et al., 2018]. Before MALDI-TOF-TOF acquisition, samples were treated with 2,4-diphenylpyranylium, which efficiently derivatizes primary amines in general, and T_1AM in particular, and can be used as a reactive MALDI-MS matrix that induces derivatization and desorption. Exogenous T_1AM was detected, whereas no endogenous T_1AM was found in sections from not-administrated mice (figure 10).

C. Monoiodotyrosine and Diiodotyrosine

In the past, MIT and DIT assay has attracted little interest. A breakthrough was the discovery that MIT and DIT are the substrates of a specific iodotyrosine dehalogenase enzyme (DEHAL-1), and that DEHAL-1 deficiency induces iodine wasting. Several genetic defects of this enzyme, as well as its inhibition by xenobiotics, such as some common air pollutants, are known to be associated with primary hypothyroidism [Moreno et al., 2008]. So, MIT and DIT assay is under consideration as a clinical test in neonatal screening and/or in the evaluation of potential endocrine disruptors.

Afink *et al.*, developed and validated a LC-MS/MS method to quantify MIT and DIT as butyl esters, with LLODs of 0.2 nM for both analytes in water, and in the range 0.2-2 nM in urine, depending on the extent of suppression effect [Afink et al., 2008]. The comparison between patients with genetic DEHAL-1 deficiency and 24 healthy adult subjects without thyroid diseases, selected as the control group, revealed significantly higher concentrations of MIT and DIT in the patients, with concentration of 100.8 and 220.8 nM for MIT, and 31.2 and 108.2 nM for DIT, against average control values 2.6±1.5 nM and 0.5±0.1 nM, respectively. Burniat *et al.* detected urinary MIT and DIT levels with the same LC-MS/MS method [Burniat et al., 2012]. They analyzed urine samples from a different consanguineous Moroccan family, and found higher concentrations of MIT and DIT in DEHAL-1 deficient subjects (ranging 74.8 and 55.2 nM) compared to control and heterozygotes subjects. The limitation of the method designed by Afink *et al*. is related to the internal standard used for the quantification. In fact, they used 3-chloro-L-tyrosine as an internal standard instead of stable isotope labelled molecules, which, perhaps, were not commercially available. Recently, Borsò *et al.* [Borsò et al., 2019] developed a HPLC-MS/MS method to quantify MIT and DIT together with TH in plasma and urine, and modified the method proposed by Saba *et al.* [Saba et al., 2014]. Briefly, the method made use of 100 µL of plasma or urine, which were added with stable isotope-labeled internal standards, namely $^{13}C_9$-MIT and $^{13}C_9$-DIT. Cold acetone was used to precipitate proteins, and the resulting supernatants were evaporated to dryness under a gentle stream of nitrogen. The dried residues were derivatized with 3.0 N hydrochloric acid in n-butanol to form the corresponding butyl esters, which were submitted to SPE. After evaporation and reconstitution with acetonitrile-HCl 0.1 M (50:50 by volume), the samples were injected into the HPLC-MS/MS system for analysis. A representative SRM chromatogram in shown in figure 11. The method showed good linearity for both MIT and DIT within the concentration range of interest, with an accuracy that ranged between 84-113%. Instrumental LLOD were 0.16 and 0.06 nM, whereas LLOQ was 0.32 nM for MIT and 0.23 nM for DIT, which were suitable for the quantification of these analytes in urine samples from DEHAL-1 knock-out mice. Although SPE was used, ion suppression was pronounced; i.e., in the range of 19-34%, but the use of stable isotope labelled internal standards, together with the high method sensitivity, overcame it [Borsò et al., 2019].

VIII. CONCLUSIONS

In recent years, mass spectrometry has firmly established itself as an indispensable analytical tool for the study of the thyroid metabolism and the diagnosis of thyroid diseases. The

possibility to setup accurate and sensitive custom methods for the quantification of a large number of analytes in different matrices, such as biological fluids, tissues, and cells, has made possible to understand physiological and physiopathological mechanisms and to investigate the role of putative new biomarkers of disease, such as T_1AM. In this review we highlighted these aspects and suggested the diagnostic importance of some metabolites, which currently are not monitored on a routine basis for the lack of commercial immunoassay-based test for the clinical use. With a great effort, mass spectrometry methods, mainly based on liquid chromatography-tandem mass spectrometry (LC-MS/MS), have entered the endocrine diagnostics, limited to the quantification of free and total T_4 and T_3 in serum and plasma, but so far mass spectrometry has not replaced traditional immunoassays. Thus, despite the favorable prospects, a further effort is still necessary to make mass spectrometry as the technique of choice for the clinical diagnostic of TH and to extend the offer to other metabolites with a clinical significance.

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ABBREVIATIONS

3,3'-T_2
3,3'-diiodothyronine

3,5-T_2
3,5-diiodothyronine

CE
capillary electrophoresis

CLIA
chemiluminescence immunoassay

CSF
cerebrospinal fluid

DEHAL-1
type1 dehalogenase

DIT
3,5-diiodotyrosine, diiodotyrosine

EI
electron ionization

ELISA
enzyme-linked immunosorbent assay

ESI
electrospray ionization

FT_3
free 3,5,3'-triiodothyronine

FT_4
free 3,5,3',5'-tetraiodothyronine

GC
gas chromatography

HPLC
high performance liquid chromatography

ICP
inductively coupled plasma

LC
liquid chromatography

LIT
linear ion trap

LLOD
lower limit of detection

LLOQ
lower limit of quantification

MIT
3-Iodotyrosine, monoiodotyrosine

MALDI
matrix-assisted laser desorption ionization

QLIT
quadrupole linear ion trap

QTOF
quadrupole time-of-flight

RIA
radioimmunoassay

RP
reversed phase

rT_3
3,3',5'-triiodothyronine, reverse T_3

SEM
standard error of the mean

SIM
selected ion monitoring

SPE
solid-phase extraction

SRM
selected reaction monitoring

T_0
thyronine

T_0AM
thyronamine

T_1AM
3-iodothyronamine

T_3
3,5,3'-triiodothyronine

T_4
3,5,3',5'-tetraiodothyronine, or thyroxine

TA_0
thyroacetic acid

TA_1
3-iodothyroacetic acid

TA_3
3,5,3'-triiodothyroacetic acid, triac

TA_4
3,5,3',5'-tetraiodothyroacetic acid, Tetrac

TBG
thyroxine-binding-globulin

Tetrac

977 3,5,3',5'-tetraiodothyroacetic acid, TA_4

978 **TH**
979 thyroid hormones, namely T_3 and T_4

980 **THM**
981 thyroid hormone metabolites
982 **TOF**
983 time-of-flight

984 **TRH**
985 thyrotropin releasing hormone

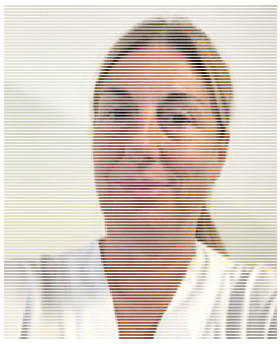
986 **Triac**
987 3,5,3'-triiodothyroacetic acid, TA_3

988 **TSH**
989 thyroid stimulating hormone

990 **UHPLC**
991 ultra-high performance liquid chromatography

BIOGRAPHIES

Marco Borsò is a PhD student at the University of Siena – doctoral programme of Biochemistry and Molecular Biology. His research interests are mainly focused on the development and application of mass spectrometry methods for the analysis of thyroid hormones in different biological matrices. He got his master's degree in Pharmaceutical Chemistry and Technology at the University of Pisa in 2016. He first approached the mass spectrometry field during his visiting master student period at the Institute of Biochemistry and Molecular Medicine in Bern, Switzerland.



Patrizia Agretti is an Executive Biologist specialist in clinical pathology. She currently works in the Chemistry and Endocrinology Laboratory of the University Hospital of Pisa where is in charge for clinical diagnostics. Her research activity is particularly focused on molecular and biochemical pathogenesis of thyroid disease and on immunometric analysis applied to the different branches of endocrinology.

Riccardo Zucchi is presently Professor of Biochemistry at the Department of Pathology of the University of Pisa. After getting an MD (1982) and a PhD (1985), he became Assistant Professor of Medicine at "S. Anna" University, in Pisa. In 2000 he moved to the University of Pisa as an Associate Professor of Biochemistry and since 2004 he is Full Professor of Biochemistry. In the first part of its career he worked on calcium homeostasis and on the biochemical basis of ischemic injury; in the last 15 years his research activity has been focused on novel thyroid hormones, particularly 3-iodothyronamine, with special reference to its assay in tissues and to the molecular basis of its cardiac, metabolic and neurological effects.

Alessandro Saba is an Associate Professor of Chemistry and Biochemistry at the Department of Pathology of the University of Pisa. His research activity is mainly focused on the use of mass spectrometry for investigations in biochemistry and clinical chemistry, with particular interest in thyroid and steroid hormone metabolism. He currently serves also as a chemical officer at the Laboratory of Clinical Pathology of the University Hospital of Pisa, where he is in charge for the clinical diagnostics with mass spectrometry on a routine basis.

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Figures

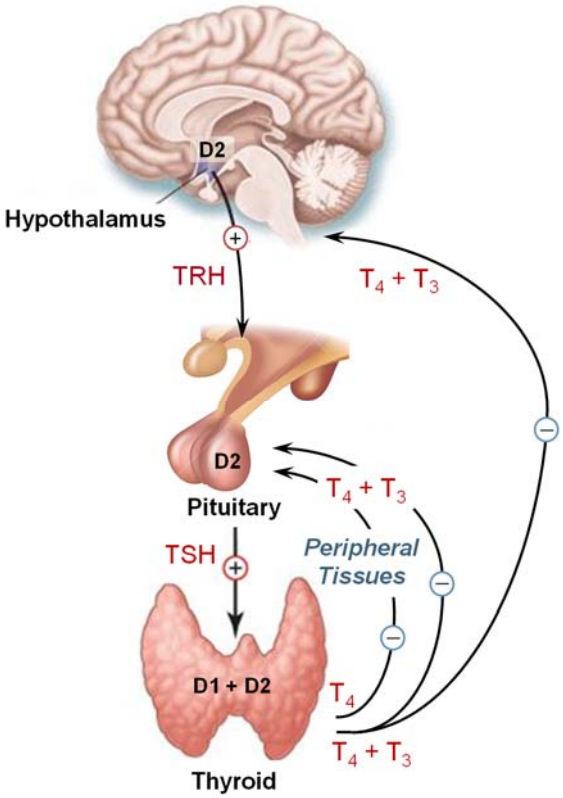

Figure 1. Diagram of the hypotalamic-pituitary-thyroid system that shows the roles of thyroxine (T_4) and triiodothyronine (T_3) in the feedback regulation of secretion of thyrotropin releasing hormone (TRH) and thyrotropin stimulating hormone TSH. Conversion of T_4 to T_3 takes place in peripheral tissues such as liver, kidney, and thyroid with type 1 iodothyronine deiodinase (D1), and thyroid, pituitary, hypothalamus, skeletal muscle, and cardiac muscle with type 2 iodothyronine deiodinase (D2).

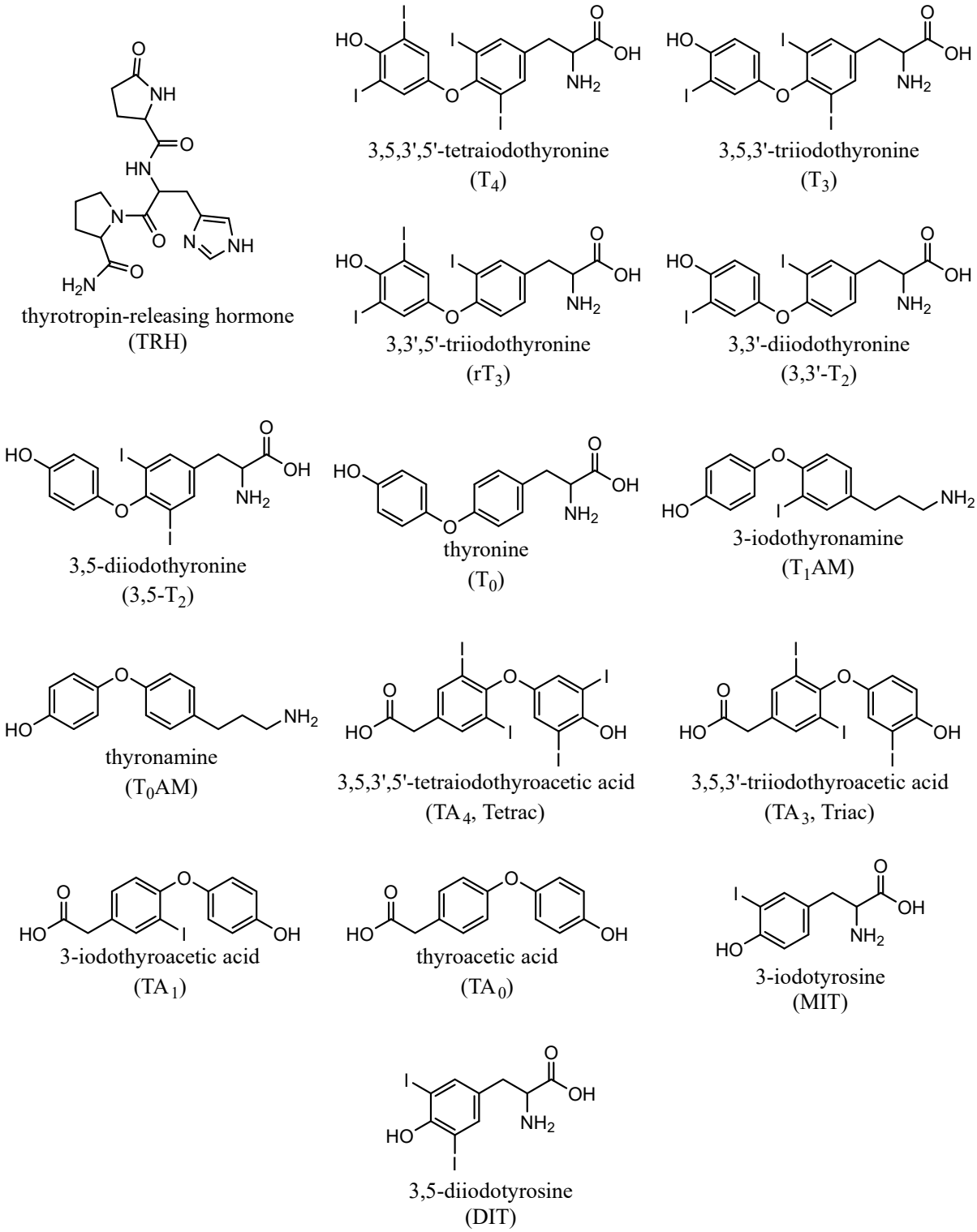

Figure 2. Chemical structures of some compounds involved in the thyroid metabolism.

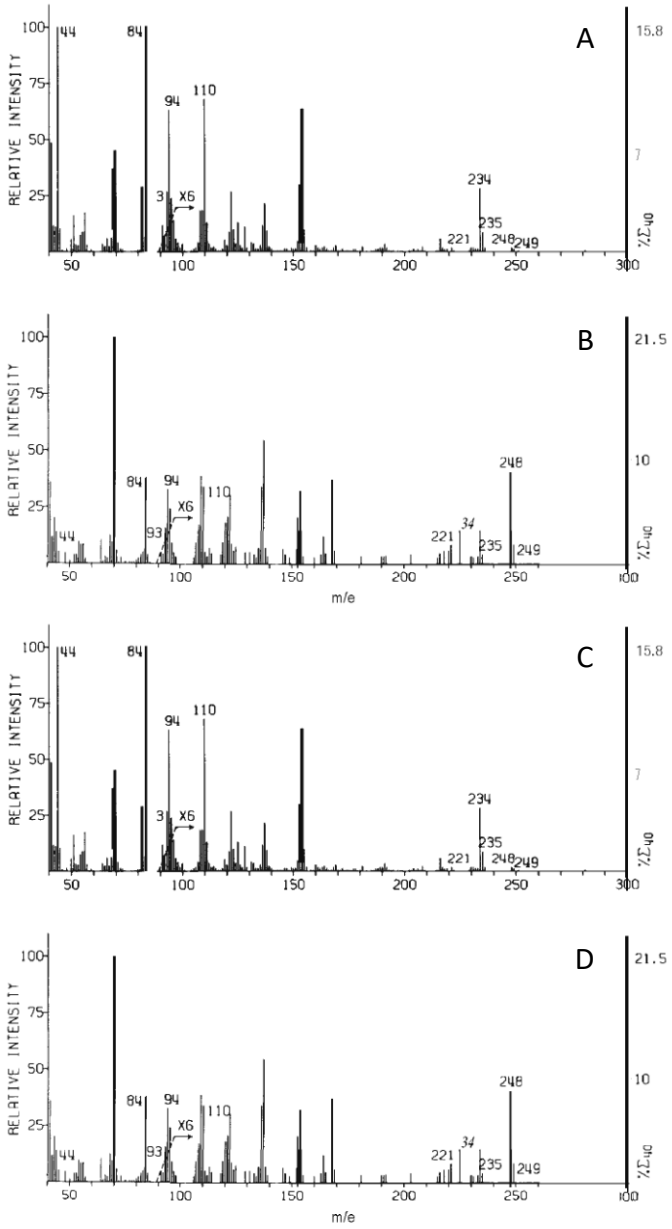

Figure 3. Low resolution mass spectra of trifluoroacetylated ovine TRF (A), trifluoroacetylated syntethic PCA-His-Pro-NH_2 (B), methylated ovine TRF (C), and methylated syntethic PCA-His-Pro-NH_2 (D). Reprinted from Guillemin R, Nobel Lecture in Physiology or Medicine 1977, with permission of The Nobel Foundation © 1977.

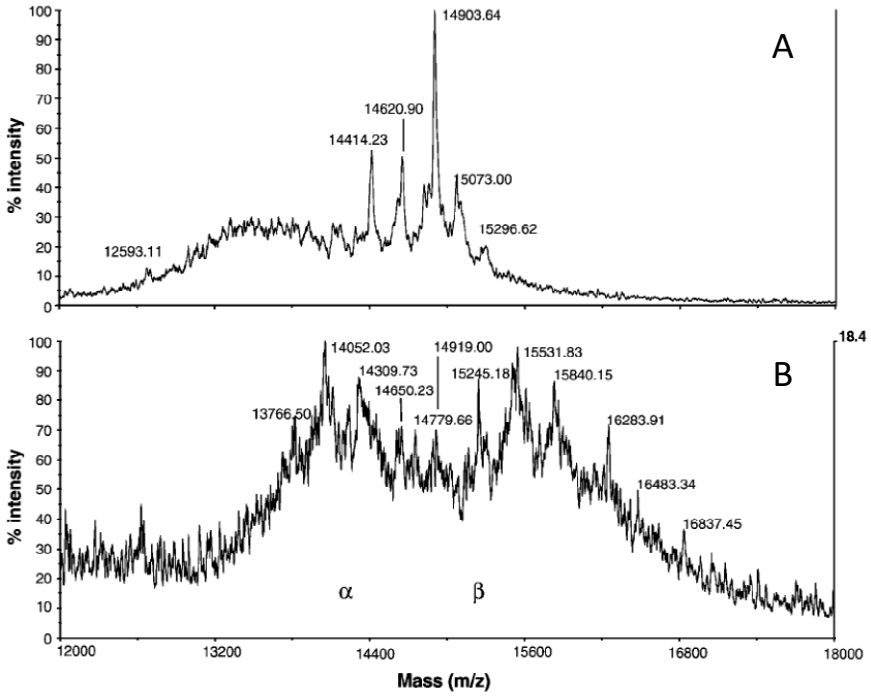

Figure 4. Qualitative mass spectra from highly purified preparation of pituitary (A) and recombinant (B) TSH, carried out with MALDI-TOF mass spectrometry in the positive-ion mode with delayed extraction. Reprinted with permission from Donadio et al., 2005. Copyright © 2005, Walter de Gruyter.

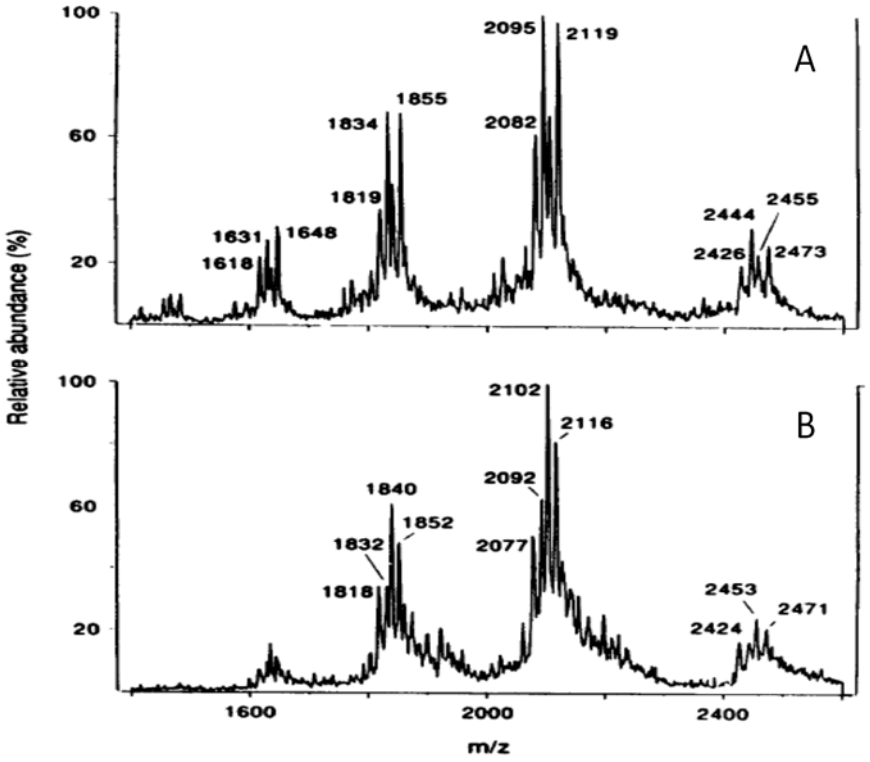

Figure 5. β-TSH characterization with LC-ESI-MS: the deconvoluted spectrum from the spectrum under chromatographic peak 1 provides (A) 14,557, 14,660, 14,727 and 14,830 g/mol as molecular masses, whereas that under chromatographic peak 2 (B) 14,542, 14,643, 14,712 and 14,815 g/mol. Reprinted with permission from Feistner et al., 1995. Copyright © 2005, John Wiley and Sons.

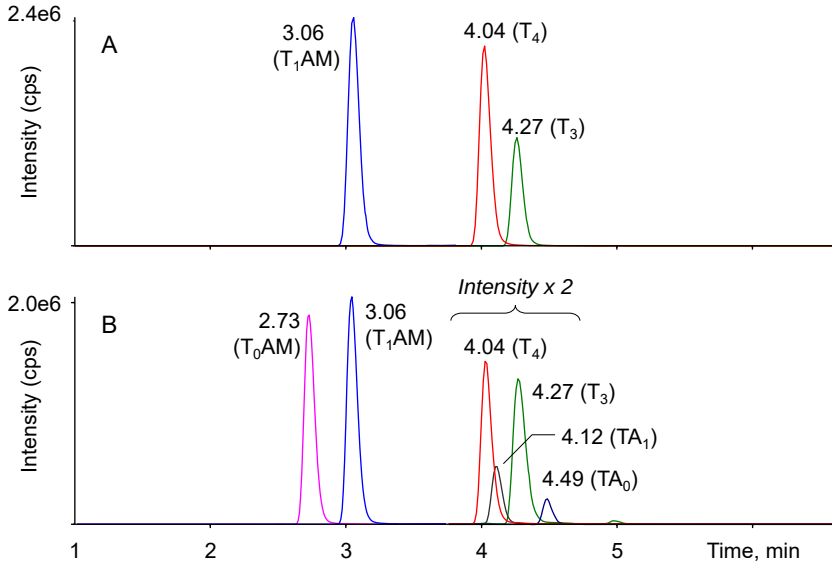

Figure 6. The two panels show representative HPLC-SRM chromatograms acquired with (A) a method that works in the positive-ion mode and with (B) a method that in the time range 0-3.5 min operates in positive-ion mode, and in the range 3.5-7.0 min in the negative-ion mode. T_3 and T_4 exhibit peaks at 4.04 and 4.27 min, T_0AM, T_1AM, TA_1, and TA_0 at 2.73, 3.06, 4.12, and 4.49 min. The peaks under the label, intensity x 2, were amplified by a factor 2 to make them more clearly visible. Concentrations of T_3 and T_4, TA_0, and TA_1 were 1 µM, whereas those of T_0AM and T_1AM were 200 nM. Adapted with permission from Saba et al., 2010. Copyright © 2010, Oxford University Press.

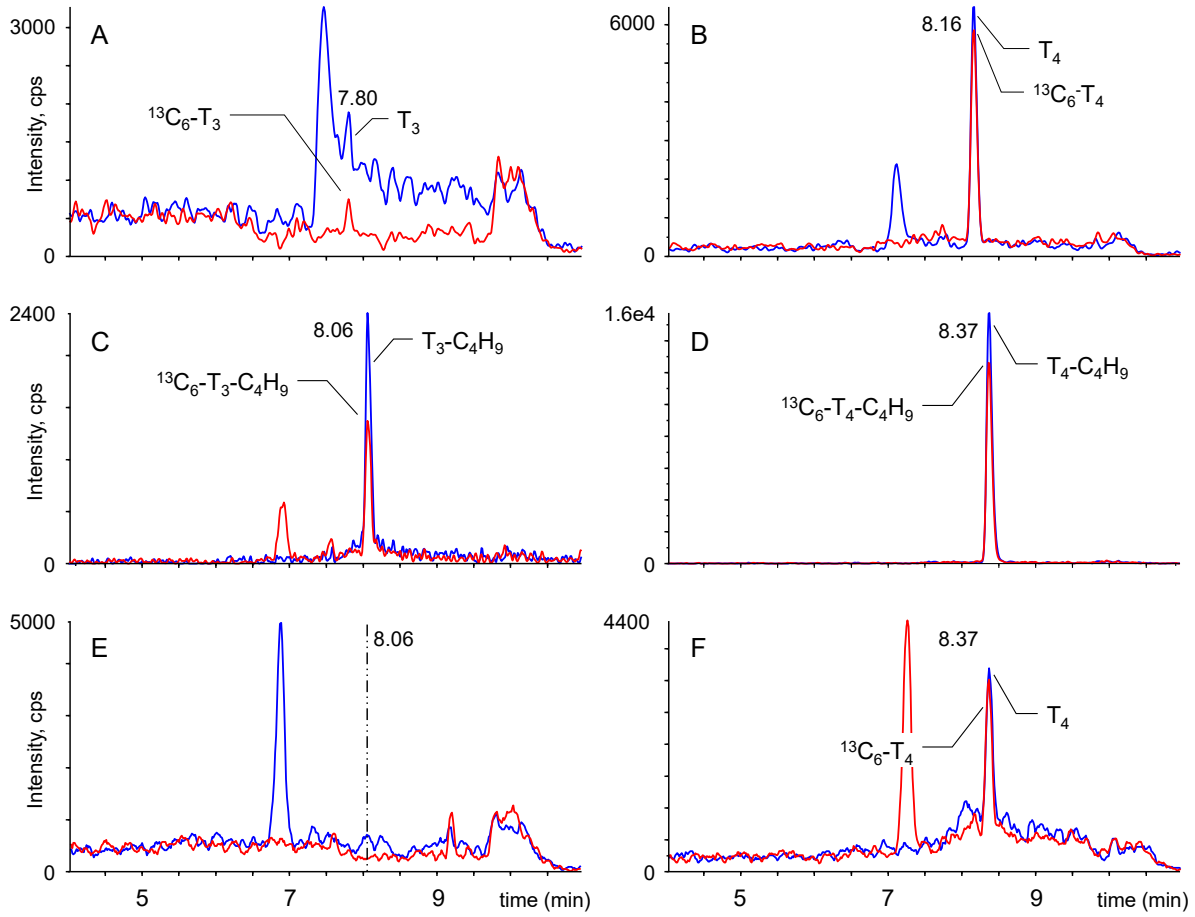

Figure 7. SRM chromatograms relative to the quantification transitions of underivatized and derivatized T_3 and T_4, from two identical aliquots of the same heart tissue: T_3 (A) and T_4 (B) from the underivatized aliquot, T_3 (C) and T_4 (D) from the derivatized aliquot, and underivatized T_3 (E) and T_4 (F) in the derivatized aliquot. Adapted with permission from Saba et al., 2014. Copyright © 2014, Georg Thieme Verlag KG.

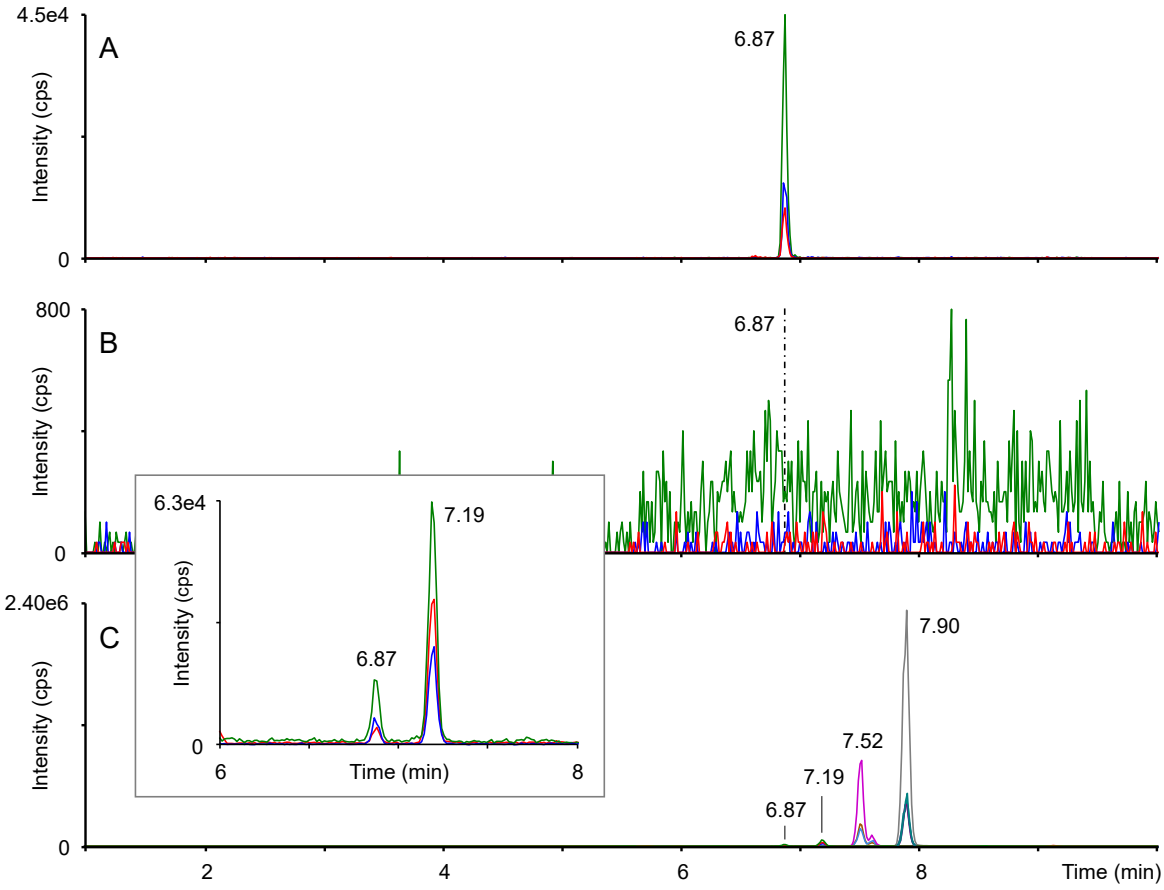

Figure 8. SRM chromatograms relative to (A) 3,5-diiodotyronine-$^{13}C_9$-^{15}N ($^{13}C_9$-^{15}N-T_2) in water solution at the same concentration of $^{13}C_9$-^{15}N-T_2 added to the human serum samples as an internal standard, (B) 3,5-diiodotyronine (3,5-T_2) as an impurity of $^{13}C_9$-^{15}N-T_2 in the same water solution, (C) a representative chromatogram from a serum sample of a healthy subject. In panel C the green, red, and blue tracings, reported also as an expanded view in the framed panels, refer to the three transitions monitored for 3, 5-T_2 (6.87 min) and 3,3′-T_2 (7.19 min); namely, *m/z* 529.9 → 352.9, 529.9 → 381.8, and 525.9 → 479.9; the three more peaks are attributable to T_3 (7.52 min), rT_3 (small peak next to T_3, at 7.61 min), and T_4 (7.90 min). Adapted from Lorenzini et al., 2019 (CC BY 4.0).

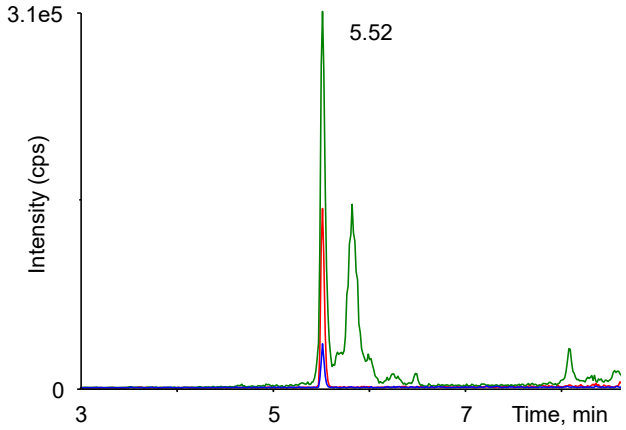

Figure 9. Endogenous T_1AM in entorhinal cortex from wild type mouse. HPLC-MS-MS tracings from a representative experiment. Transitions monitored by tandem mass spectrometry (*m/z* 356.2 → 195.2, 356.2 → 212.2, and 356.2 → 339.1) are shown by the blue, red, and green lines, respectively. Adapted from Accorroni et al., 2020. Copyright © 2020, Mary Ann Liebert, Inc.

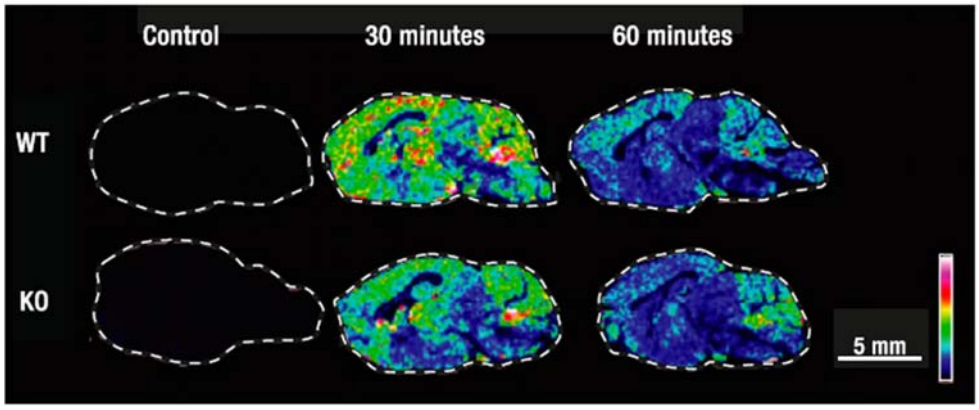

Figure 10. Distribution of T1AM in sagittal brain slices of Wild Type and Trace Amine-Associated Receptor 1 knockout mice injected with T1AM intraperitoneally at 20 mg/kg. A MALDI-TOF/TOF mass spectrometer was used to acquire MS images which are shown using a rainbow scale and normalized against the total ion count. Reprinted from Zhang et al., 2018 (CC BY 4.0).

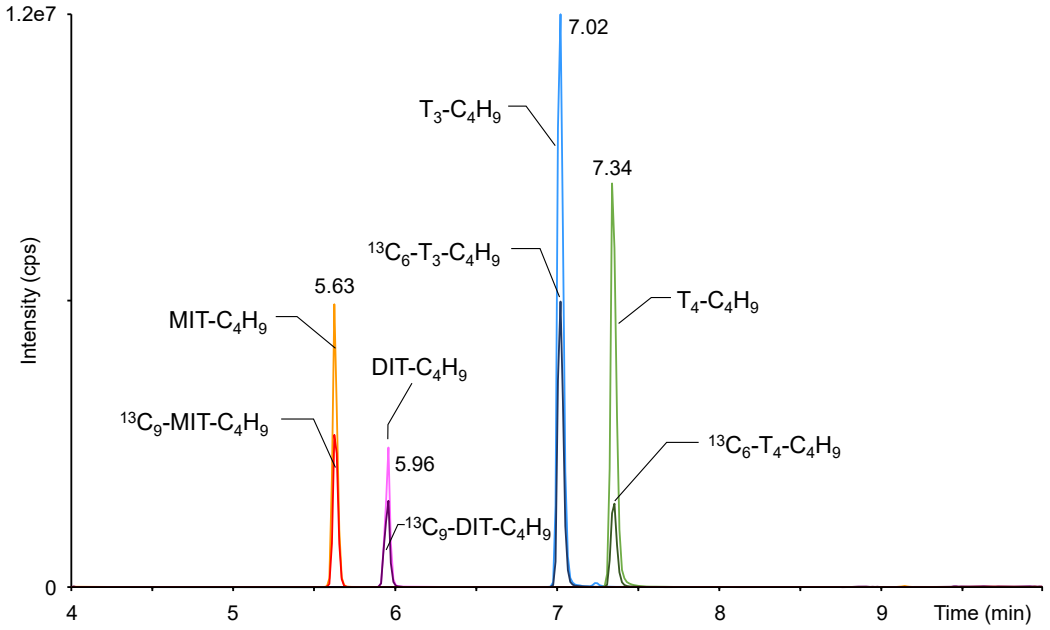

Figure 11. Chromatogram of a standard solution containing butylated MIT, DIT, T_3, T_4, and the relative stable isotope labeled internal standards. The trace of each compound was obtained by summing three SRM transitions monitored during the analysis. Adapted from Borsò et al., 2019.