Chromatographic methods coupled to mass spectrometry for the determination of oncometabolites in biological samples-A Review

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Abstract

It is now well-established that dysregulation of the tricarboxylic acid (TCA) cycle enzymes succinate dehydrogenase, fumarate hydratase, and isocitrate dehydrogenase leads to the abnormal cellular accumulation of succinate, fumarate, and 2-hydroxyglutarate, respectively, which contribute to the formation and malignant progression of numerous types of cancers. Thus, these metabolites, called oncometabolites, could potentially be useful as tumour-specific biomarkers and as therapeutic targets. For this reason, the development of analytical methodologies for the accurate identification and determination of their levels in biological matrices is an important task in the field of cancer research. Currently, hyphenated gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) techniques are the most powerful analytical tools in what concerns high sensitivity and selectivity to achieve such difficult task. In this review, we first provide a brief description of the biological formation of oncometabolites and their oncogenic properties, and then we present an overview and critical assessment of the GC-MS and LC-

MS based analytical approaches that are reported in the literature for the determination of oncometabolites in biological samples, such as biofluids, cells, and tissues. Advantages and drawbacks of these approaches will be comparatively discussed. We believe that the present review represents the first attempt to summarize the applications of these hyphenated techniques in the context of oncometabolites analysis, which may be useful to new and existing researchers in this field.

Keywords

Oncometabolites; biological samples; tumour biomarkers; liquid chromatography- mass spectrometry; gas chromatography-mass spectrometry

1. Introduction

A common feature of cancer cells is their ability to modify their metabolism to achieve and sustain the energetic and anabolic demands of their uncontrolled proliferative capacity. This reprogramming of conventional metabolism in cancer cells, compared with normal cells, is considered to be one of the hallmarks of cancer [1,2], and involves several bioenergetic and biosynthetic pathways, such as enhanced glycolysis, glutaminolysis, fatty acid metabolism, pentose phosphate pathway, and mitochondrial biogenesis [3]. Furthermore, other metabolic changes, driven by mutational deregulation of enzymes belonging to a key metabolic pathway, the tricarboxylic acid (TCA) cycle, have been shown to play a causal role in tumorigenesis, through the overproduction of particular intermediary metabolites, termed “oncometabolites” [4], which, in turn, contribute to the formation and malignant progression of certain cancers [5,6].

Given the implications of abnormal oncometabolite levels in cancer pathogenesis, they have been proposed, and are currently being evaluated, as potential clinically useful

tumour-specific biomarkers with broad applications in the early diagnosis, choice of treatment, and prediction of therapeutic response [6–9]. This is an emerging area of great interest in cancer research, that requires the application of reliable analytical methodologies to obtain accurate quantitative information about the abundance of oncometabolites in different biological samples. This quantitative information is also relevant to improve the current knowledge on the mechanisms by which oncometabolites contribute to the initiation and development of malignant phenotypes, as well as their role in chemotherapy response and, therefore, as possible targets for anticancer pharmacological interventions.

In this review we first present a short description of the biological formation of oncometabolites and their oncogenic properties [4–6], with the goal of introducing non-experts to the concepts motivating ongoing research. Then we focus on summarizing, comparing and critically discussing the main chromatographic-MS based analytical methodologies available for the determination of oncometabolites in different biological samples, such as biofluids, tissues and cells. To our knowledge, this is the first review about this topic. Based on these considerations, we believe that the present review will improve the existing literature, what may be helpful in future research in this field, and in clinical handling of patients, considering the clinical usefulness of oncometabolites determination in biological samples.

2. Biological formation of oncometabolites.

The term oncometabolite is relatively new and refers to small molecules, intermediates of normal metabolism, with putative oncogenic properties, and whose abundance is significantly increased in cancer cells relative to normal ones [4]. To date, the most common and studied oncometabolites are succinate, fumarate, and 2-hydroxyglutarate [4–6] (see structure in Figure 1), that are generated in excess due to mutations in the genes encoding

the TCA cycle enzymes succinate dehydrogenase (SDH), fumarate hydratase (FH) and isocitrate dehydrogenase 1 and 2 (IDH1/2), respectively [5] (Fig. 1). These enzymes share metabolic proximity in the TCA cycle, also known as Krebs' cycle or citric acid cycle, which consists of a series of enzyme-catalyzed reactions, occurring in the mitochondria, that allow mammalian cells to obtain energy to synthesize macromolecules, and to achieve redox balance.

The SDH enzyme was the first mitochondrial enzyme whose genes were found to be mutated in cancer [10]. This enzyme, which is composed of four subunits, acts in the TCA cycle by catalyzing the reversible redox transformation of succinate to fumarate (see Fig. 1). Mutations in genes that encode any one of the SDH subunits significantly reduce its enzymatic activity, leading to an increase in the cellular concentration of succinate from about 5 μM levels (physiological concentration) to millimolar levels [6], which is sufficient to drive the neoplastic transformation of cells [5,6]. These mutations are commonly found in hereditary paraganglioma and pheochromocytoma [5,11,12], but have also been identified in other types of tumours such as gastrointestinal stromal tumours, renal cell carcinomas [5,11,12], thyroid tumours, testicular seminoma, neuroblastoma [6,10,12], and T-Cell leukaemia [6].

Another deregulated TCA cycle enzyme in cancer is FH, that exists as a homo-tetrameric protein present in two isoforms coded by the same gene, a cytosolic isoform and a mitochondrial isoform. The mitochondrial isoform catalyzes the stereospecific and reversible hydration of fumarate to malate in the TCA cycle (see Fig. 1). Mutations in the gene encoding FH have been found to decrease FH activity with the consequent accumulation of fumarate. As in the case of succinate, fumarate is present in normal cells at micromolar levels, and inhibition of FH functionality leads to an abnormal accumulation of fumarate to millimolar levels [6], which has the potential to initiate and drive oncogenesis

[13]. Evidence suggests that mutations in FH gene predispose to multiple cutaneous and uterine leiomyomas [6,11], as well as, to hereditary leiomyomatosis and renal cell cancer [5,6,11], and may also be involved in the pathogenesis of breast, bladder and testicular cancers [11]. Mutations affecting the FH gene are also found in a subset of paragangliomas and pheochromocytomas [5,7].

Finally, the oncometabolite 2-hydroxyglutarate (2HG), which was the first oncometabolite to be discovered, is formed by mutational deregulation of the IDH enzyme, a homodimeric protein that consists of three isoforms, IDH1, localized in the cytosol, and IDH2 and IDH3, present in the mitochondria. In the TCA cycle, IDH catalyzes the reversible oxidative decarboxylation of isocitrate to alpha-ketoglutarate (α-KG) (see Fig. 1). Mutations in genes encoding the IDH1 and IDH2 isoforms result in the gain of a new enzymatic activity that allows the catalytic reduction of α-KG to the D enantiomeric form of 2HG (D-2HG) [14,15]. 2HG is a poorly characterized metabolite, naturally present as two enantiomers, D-2HG and L-2HG, which are unwanted by-products of cellular metabolism, and whose intracellular levels are maintained very low due to the action of endogenous enzymes that catalyze their conversion to α-KG [16]. However, D-2HG accumulates to supraphysiological levels within cells with mutations affecting IDH1/2 genes [14,17,18], and this accumulation has a drastic effect on cellular processes that promote tumorigenesis [18]. These mutations occur in multiple human cancers, including low-grade glioma, secondary glioblastoma, acute myeloid leukaemia (AML), chondrosarcoma [4,5,11,19] and cholangiocarcinoma [4,7,19]. Mutations in genes encoding IDH1/2 have also been identified in other malignant tumours, such as angioimmunoblastic T-cell lymphomas, thyroid, colon, prostate [6,11], brain and cartilaginous cancers [6].

3. Oncogenic properties of oncometabolites

The brief description given above shows that many types of tumours are associated with aberrant levels of oncometabolites. However, the mechanisms by which an oncometabolite may exert effects on cell functions that promote carcinogenesis are still not completely understood. The three oncometabolites are structurally similar to α-KG, and act as its antagonists to competitively inhibit the activity of multiple α-KG-dependent dioxygenase enzymes, which contribute to tumorigenesis via activation of the pseudohypoxia pathway [4,20] and epigenetic dysregulation [4,5,21]. Specifically, it has been proposed that these oncometabolites inhibit prolyl hydroxylase enzymes [4,20,22], which are involved in the degradation of the hypoxia-inducible factor-1α (HIF-1α), a transcription factor that accumulates in response to decreased cellular oxygen levels to facilitate tumour growth. Thus, inhibition of these enzymes leads to HIF-1α stabilization in the presence of oxygen [4,20,22], a condition known as pseudohypoxia. This aberrant increase in HIF-1α levels regulates the transcription of hundreds of genes implicated in a myriad of functions, including the promotion of angiogenesis, erythropoiesis, cell growth and proliferation, invasion/metastasis, and metabolic adaptation [23].

Oncometabolites are also potent inhibitors of other α-KG-dependent dioxygenases enzymes, such as the Jumonji C-terminal domain family of histone demethylases, and the ten-eleven translocation family of DNA hydroxylases [4,20,24,25], that mediate the demethylation of histone and DNA, respectively [5]. Thus, oncometabolite accumulation leads to genome-wide hypermethylation of both histone and DNA [4,6,24,25], which are very important epigenetic changes (chemical modifications of histone and DNA that affect gene expression without changing the DNA sequence) generating widespread deregulation of gene expression [26]. These aberrant histone and DNA methylation patterns induced by oncometabolites are crucial in tumorigenesis because of their potential role in silencing

tumour suppressor genes and/or activating oncogenes [26], and also in modifying chromatin structure [25].

In summary, currently there is a large body of evidence indicating that the main oncogenic mechanism shared by the three oncometabolites is the inhibition of the α-KG-dependent dioxygenases enzymes. In addition, there are also emerging findings showing that each oncometabolite exerts other distinct, and specific, oncogenic functions [6,7], which can explain the different types of tumours associated with their accumulation [5,7].

4. Analytical techniques for the determination of oncometabolites

Metabolomics is a research field that investigates the complex and diverse metabolic processes in biological systems. Depending on the purpose of the research, there are two main types of metabolomic strategies: untargeted metabolomic and targeted metabolomic analyses. Untargeted metabolomics focuses on the measurement of all the detectable metabolites in a single sample, including unknown chemicals, generating a metabolomic fingerprint. By contrast, targeted metabolomics involves the measurement of a pre-defined small set of known metabolites, such as oncometabolites.

During the past two decades, several analytical approaches have been developed for the targeted accurate identification and determination of trace amounts of oncometabolites in biological samples, such as biofluids, tissues and cells. These approaches are mainly based on nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS) techniques, which can offer adequate analytical performance characteristics to achieve such difficult task. NMR spectroscopy and MS have evolved as the most common techniques in both untargeted and targeted metabolomic studies, due to their high sensitivity and/or selectivity, which are necessary to identify and quantify known and unknown metabolites in

complex biological matrices, although both techniques present their own advantages and disadvantages [27–29].

NMR spectroscopy is considerably less sensitive and selective than MS, but it requires minimal sample preparation, and it has the potential for the *in vitro*, or *in vivo*, non-invasive imaging of metabolites in cells and/or tissues. NMR spectroscopy-based methods have been widely employed for *in vivo* detection/quantification of 2HG in IDH-mutant glioma patients [30–37], and succinate in SDH deficient paragangliomas patients [38,39], as well as, in patients with other suspected SDH-related tumours [40].

Unlike NMR spectroscopy, MS has high sensitivity and selectivity, especially when it is used coupled to a separation technique, such as liquid chromatography (LC) or gas chromatography (GC). These hyphenated LC-MS and GC-MS approaches, in combination with an effective sample preparation procedure, are currently the most powerful analytical tools for targeted metabolomics in the field of cancer research [28,41].

Mass spectrometers operate by ion formation, separation of ions according to their mass-to charge (*m/z*) ratio, and detection of separated ions. Currently, chromatography-MS based technologies offer a variety of choices for chromatographic separation, ionization, and mass spectrometric analysis, that differ in operational principles and performance characteristics [42,43]. In GC-MS, ionization is usually achieved by using electron impact (EI), while in LC-MS the most common ionization mode is electrospray ionization (ESI). A range of mass analyzers may be used, including quadrupoles and ion traps, which offer good sensitivity but limited resolving power, or higher mass-resolution analyzers, such as time-of-flight (TOF), Fourier transform ion cyclotron resonance (FTICR) or Orbitrap. However, the reliability and affordable cost of single quadrupole (Q) instruments have made them the most popular and robust option in metabolomics research [42,43]. Moreover, they can also be arranged in a tandem configuration, such as a triple quadrupole mass spectrometer (QqQ),

which enable the use of tandem MS (MS/MS, or even MS^n) methods to overcome the challenging identification and quantification problems associated with co-eluting analytes in complex matrices, specially, in LC-MS based approaches [42,43].

Analytical strategies based on LC-MS and GC-MS techniques, applied to targeted metabolomic studies, involve a methodological workflow, shown in Figure 2**,** which consists of several steps, including sample collection, sample preparation, data acquisition (metabolite separation/detection by GC-MS or LC-MS), data processing, validation and, finally, biochemical interpretations of the obtained results [44]. Sample preparation constitutes a crucial step in this methodological workflow since it may affect metabolite content, data quality and interpretation of any obtained results. The choice of an adequate sample preparation procedure mainly depends on the sample type and volume, the physicochemical properties of the measured metabolites and the analytical technique used for sample analysis. Sample preparation of biological samples for the determination of polar metabolites, such as oncometabolites, by chromatographic-MS based techniques usually entails the selective extraction and enrichment of the targeted metabolites from the sample (e.g. serum, tissues, cells), while minimizing the extraction of lipids, proteins and other matrix components. This may be usually achieved using different extraction procedures, that vary depending on the nature of the extraction reagent (e.g. perchloric acid, trichloroacetic acid, methanol, acetonitrile, ethyl acetate), the composition of solvent mixtures (e.g. 80% (v/v) methanol/water, 70% (v/v) acetonitrile/water, methanol/chloroform (3:1)), the added acid or alkali, and the temperature of extraction. Prior to metabolite extraction, a rapid chilling/quenching of the metabolic activity in tissues and cells samples is necessary. This is traditionally achieved by the instant change of sample temperature, to either low (e.g. < -40 ^oC) or high temperatures (e.g. > 80 ^oC), or by changing the sample pH to extreme ones, either alkali (e.g. adding KOH or NaOH) or acid pH (e.g. adding perchloric or trichloroacetic

acids). This step may also be integrated with the subsequent metabolite extraction step, for instance using 80% (v/v) methanol/water at -80 ^{o}C. Finally, after extraction, solvents should be readily removed, e.g. by lyophilization, precipitation or evaporation, prior to the analysis of metabolites using chromatographic-MS based techniques.

In the subsequent sections, we present an overview, and critical assessment, of the GC-MS and LC-MS based analytical approaches that are reported in the literature for the determination of oncometabolites in samples of biological origin. Advantages and drawbacks of these approaches will be comparatively discussed.

4.1. GC-MS-based methodologies

The coupling of capillary GC to MS (GC-MS), with EI ionization, is the most mature technique for the determination of small molecular weight metabolites [45,46], due to its robustness, high separation efficiency, high selectivity and reproducibility, and the large number of mass spectral databases/libraries available for identification of metabolites of interest [41,45]. In fact, the technique has been utilized since the 1970s in clinical laboratories, for the simultaneous analysis of urinary organic acids, including 2-hydroxyglutaric, fumaric and succinic acids, for the diagnosis of organic acidurias [45], a heterogeneous group of inborn errors of metabolism in which excessive organic acids are excreted in the urine.

Since oncometabolites (short chain dicarboxylic acids, see structure in Fig. 1) are polar and non-volatile, they need to be derivatized to increase their volatility, to make them amenable for GC–MS determination. The most common derivatization procedure is the silylation of the carboxylic groups, using well-stablished protocols and different commercially available reagents, such as N-methyl-N-(trimethylsilyl)heptafluorobutyramide (MSHFBA [47–50], N-methyl-N-(tertbutyldimethylsilyl)trifluoroacetamide (MTBSTFA) [51–55], N-methyl-N-

(trimethylsilyl)trifluoroacetamide (MSTFA) [56,57], and N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) [58]. In some cases, tert-butyldimethylchlorosilane (TBDMCS) [59–61], or trimethylchlorosilane (TMCS) [62–67], are added to the silylation reactions to accelerate the process. Using these derivatization reagents, several GC-MS based methodologies, which are summarized in Table 1, have been developed and applied to the determination of oncometabolites in different biological samples.

Sample preparation approaches, prior derivatization and GC-MS analysis, are generally one-step processes consisting of simultaneous metabolic quenching and extraction of targeted metabolites, or only extraction, using different solvent systems, such as ethyl acetate [49,50,65], methanol [58],methanol at −20 °C [63]; 80% (v/v) methanol/water at −80 °C [51–55], 95% (v/v) methanol/water [66], 50% (v/v) acetonitrile/water [60], 70% (v/v) acetonitrile/water [59], perchloric acid [61], methanol/water/chloroform (2.5:1:1) [56], methanol/chloroform (1:2) [57], and methanol/chloroform (3:1) [64]. In a few cases, the quenching step, e.g. with 60% (v/v) aqueous methanol buffered with 70 mM of HEPES at −50 °C, is carried out before metabolite extraction, e.g. with 75% (v/v) aqueous methanol/methyl tert-butyl ether (9:1) [67].OJOOOO Additional clean-up of sample extracts, after protein fraction removal by precipitation, is sometimes required [51–53,60,63]. This is carried out by solid phase extraction of anionic targeted oncometabolites, using an AG-1×8 100–200 anion exchange resin [51–53], by chloroform extraction of non-polar compounds [63], or by sequential extractions of non-polar compounds with diethyl ether and of polar compounds with ethyl acetate [60].

Separation and detection of oncometabolites by GC-MS is mostly carried out by using general purpose nonpolar 5% phenyl/95% dimethyl polysiloxane capillary columns (DB-5MS, HP-5MS, HP Ultra-2, VF-5 ms, Sapines-5MS+), coupled with single quadrupole electron ionization MS (EI-qMS), operating in the single ion monitoring (SIM) mode to achieve good

sensitivity (Table 1). Although quantification has been performed by using the external standards calibration method [51–53,55,57,58], accurate quantification usually requires the use of the internal standard calibration method with stable isotopically labelled (e.g. 3-hydroxyglutaric acid-d4, mystiric acid-d27, fumaric acid-^{13}C4, succinic acid-^{13}C4, glutamic acid-^{15}N, 2HG-^{13}C5, mystiric acid-^{13}C2) [48,50,61–63,65,67], or non-labelled (e.g. heptadecanoic acid, D,L-norleucine, 3,4-dimethoxybenzoic acid, 2-isopropylmalic acid, L-2-chlorophenylalanine) [54,56,59,60,64,66], internal standards, which are typically added to the sample prior the extraction, to correct for analyte losses during all the analytical procedure.

As shown in Table 1, most of the existing GC-MS methodologies focus on the targeted determination of only 2HG levels in different biological samples, including serum from acute myeloid leukaemia (AML) [47,50,64] and glioma [49] patients, and healthy people [64,65]; tumour tissues from glioma [48,55], breast cancer [62], and head and neck squamous cell carcinoma (HNSCC) [58] patients; and cultured cancer cells [51–54,57]. Although a publication reports the simultaneous GC-MS quantification of fumarate and succinate in tumour tissues from pheochromocytomas (PHEOs)/paragangliomas (PGLs) patients [61], these two oncometabolites are usually determined together with other glycolysis and TCA cycle metabolites, e.g. in serum from obese mice [59], and cardiovascular diseases patients [63], in cerebrospinal fluid (CSF) from glioma patients [56], in liver tissue from obese mice [59], in tumour tissues from gastric adenocarcinoma patients [60], and in hepatic cell lines [67]. There is also a publication dealing with the simultaneous GC-MS quantification of 2HG and the TCA cycle metabolites, including fumarate and succinate, in serum from nasopharyngeal cancer (NPC) patients [66].

The aim of most of the reported GC-MS studies are to explore the clinical usefulness of oncometabolites, as biomarkers of oncometabolite-related cancer activity [47–

50,55,61,64], to study the potential value of 2HG in identifying IDH-mutated tumours [47–55,64], or its role in tumorigenesis [57,58,62], and to identify possible metabolic markers in different types of cancers [60,66]. Therefore, in these studies the analysis of the targeted oncometabolites is performed using well-established GC-MS approaches, which have not been specifically fully validated for the determination of oncometabolites in biological samples. Moreover, information on the analytical characteristics of the employed GC-MS methods is not reported, which makes their critical comparison very difficult.

Considering the reported results, in some of these studies [47,50,64], AML patients with IDH mutations present increased serum levels of 2HG compared to AML patients without IDH mutations (ranges: 54.7–870.9 vs 1.0–15.8 μM [47], 4.2-492 vs 0.9-20.8 μM [50], and 29.8-56.7 vs 23.6-25 μM [64]), and also compared to normal control individuals (27,2 ± 7 μM [64]). These results provide a rationale for serum 2HG measurements to be used in the clinical setting as a tool to identify AML patients with IDH mutations. In contrast, relationships between 2HG levels and IDH mutations in glioma patients are not so clear because, whereas accumulation of 2HG was detected in IDH mutant tumours tissues [48,55], significant differences of serum 2HG levels were no detected between a small cohort of IDH mutant (n= 19) and IDH wild type (n= 18) glioma patients [49]. High tissue 2HG concentrations (1–20 mmol/kg) have also been described in a subgroup of breast tumours that had poor clinical outcome [62], and in HNSCC carcinoma [58], despite the absence of IDH mutations in both types of tumours. With respect to fumarate and succinate, the succinate-to-fumarate ratio in tumour tissues may be used as a new metabolic marker for SDH-related PHEOs/PGLs, because it was higher in SDH-related PGLs than in apparently sporadic PHEOs/PGLs [61]. Furthermore, the levels of fumarate and succinate, together with other organic acids of the TCA cycle and glycolysis, were reported to be significantly increased in gastric cancer tissues

compared to normal ones [60]; while no differences were found in the levels of these metabolites in CSF samples from patients with gliomas of different malignancy [56].

Relatively few publications have reported the development and validation of GC-MS methods for the determination of oncometabolites in biological samples, such as serum [59,63,65,66], tissues [59] and culture cells [67]. Among them, a GC-EI-qMS method with BSTFA plus 1% TMCS derivatization, was specifically devised and validated for measuring 2HG serum levels in routine clinical laboratories, and its applicability was evaluated analysing serum from healthy people and five AML patients [65]. The same derivatization reagent was used to develop and validate a GC-EI-qMS method for the simultaneous determination of 2HG and all TCA cycle metabolites in serum from nasopharyngeal carcinoma (NPC) patients, to explore their clinical significance in this cancer [66]. A GC-EI-ion trap-MS method, using derivatization with BSTFA plus 2% TMCS, was developed and validated to investigate the potential value of serum TCA cycle metabolites levels as biomarkers of cardiovascular diseases [63]. Derivatization with MTBSTFA, containing 1% TBDMCS, was used to develop and validate a GC-EI-qMS method to quantify 13 energy metabolites, including all the TCA cycle intermediates, in liver tissue and serum from high-fat diet-fed obese mice, to explore their role in obesity development [59]. Recently, different sample preparation procedures, sylilation reagents and chromatographic conditions were assayed to develop and validate a GC-EI-QqQ-MS/MS method, working in selected reaction monitoring (SRM) mode, for the simultaneous determination of several primary metabolites in glycolysis and TCA cycle, including fumarate and succinate [67]. The final working conditions are presented in Table 1. The developed method was successfully applied to the analysing of three kinds of hepatic cell lines [67].

All these methods were validated using international accepted guidelines for parameters, such as linearity, intra- and inter- day precision, recovery, limit of detection

(LOD) and limit of quantification (LOQ), among others. The values obtained for the main validation parameters are comparatively summarized in Table 2. As can be seen, LOQs in the low μM range have been reported for the determination of the three oncometabolites in serum, e.g. 0.0675-0.243 μM for 2HG [65,66], 0.0565-0.5 μM for succinate [59,63,66], and 0.0575 -0.5 μM for fumarate [59,63,66]. Low LOQs are also obtained for the determination of succinate and fumarate in tissues, e.g 0.5 μM [59], and in culture cells, e.g. 1.32 and 0.168 μM, respectively [67]. These LOQs are low enough to determine oncometabolites in these types of biological samples. The intra- and inter-day precision of these methods, expressed as percent of relative standard deviation, is acceptable (below 20%), and the recoveries are satisfactory.

4.2. LC-MS-based methodologies

Although GC-MS is the oldest technique used in targeted metabolomic studies, LC-MS with electrospray ionization (ESI) has the advantages that it is able to determine a larger number of intact metabolites with no need for chemical derivatization, and different chromatographic modes can be selected for separation [42,43]. In addition, the advances in MS and interface technologies, and in LC techniques, such as the development of fast and more efficient ultra-high-performance liquid chromatography (UHPLC), have resulted in greatly improved selectivity and sensitivity [29,42,43]. Thus, LC-MS-based approaches are increasingly used in the metabolomics field. These approaches frequently employ tandem mass spectrometry (MS/MS), using a QqQ instrument, which benefits from increased sensitivity and specificity using SRM or MRM modes for quantification.

Several LC-MS methods, which are summarised in Table 3, have been reported for the detection and determination of oncometabolites in biological samples using different LC separation mechanism, including ion-exchange [68,69], reversed-phase [70–73], and ion

pairing-reversed phase [14,74–82], coupled with negative ESI(-)-MS. Targeted quantitative analysis is performed via MRM mode, on QqQ-MS/MS instruments, in the majority of the published methods (Table 3). Quantification is mostly based on the application of external standard calibration [14,77–82], or internal external calibration methods [68–70,72–74,76], using stable isotope-labelled standards (e.g. 2HG-^{13}C5, 2HG-d3, fumarate-^{13}C4, fumarate-^{13}C2-d2, succinate-^{13}C4, succinate-d6, pentanoic acid-d4) to correct for recoveries. The use of matrix-matched calibration [71], or the standard addition method [75], has also been reported.

Regarding sample preparation of biological samples prior LC-MS analysis, several quite simple extraction procedures are used, which are mainly based on the simultaneous stop of metabolic activity and protein precipitation, or only protein precipitation, using different reagents, such as 5% trichloroacetic acid (TCA) [71], cold methanol [74], 80% (v/v) methanol/water at – 80 ºC [14,77–82], methanol [68,70], 80% (v/v) methanol/water [70], 1% formic acid in acetonitrile [72], and acetonitrile [69,76]. In some cases, quenching, e.g with ice-cold methanol, is carried out before protein precipitation, e.g. with ice-cold 80% (v/v) methanol/water [73].OJOOOOOO A more complicated sample preparation procedure, which involves stop of metabolic activity and protein precipitation with 60% (v/v) methanol/water at – 50 ºC, followed by supernatant treatment with a mixture of 60% (v/v) aqueous methanol at – 20 ºC/0.3 M KOH in 25% (v/v) aqueous ethanol (1:4), is necessary if the analysis is focus on the determination of oncometabolites together with several other important metabolites [75].

As in the case of GC-MS methodologies (Table 1), most of the LC-MS publications, summarized in Table 3, focus on the targeted determination of only 2HG in different biological samples, such as CSF [77], serum and urine [76,77] from glioma patients; urine from breast adenocarcinoma [68], and AML [79,80] patients; serum from breast adenocarcinoma [68],

intrahepatic cholangiocarcinoma [69] and AML [78–81] patients; bone marrow aspirates from AML patients [79,80,82]; and AML cell lines [78]. The other two oncometabolites, fumarate and succinate, are usually determined together with other TCA cycle metabolites in different biological samples, including tumour tissues from PHEOs/PGLS patients [70], mouse liver tissue [72], human plasma and serum from healthy donors [71,72], cell lines [72], and cells extracts from *Escherichia coli* [75]. In addition, some methods report the simultaneous determination of 2HG and TCA cycle metabolites, including fumarate and succinate, in tumour tissues from glioma patients [14,73,74].

Considering the anionic nature of oncometabolites, ion-exchange chromatography coupled to MS is a valuable tool for the separation of these compounds. However, the main challenges of interfacing this chromatographic mode and ESI-MS are the compatibilities in mobile phase composition, because the eluent choice in ESI-MS is severally restricted to volatile compounds and low salt content. Thus, only two publications, from the same research group, have reported the application of an ion exchange-based LC-MS/MS method, using a Bio-Rad Fast Acid Analysis column and volatile 0.1% (v/v) formic acid/water as mobile phase, for the determination of 2HG in serum and urine from IDH1-mutant and IDH-wild-type breast adenocarcinoma patients [68], and serum from intrahepatic cholangiocarcinoma (ICC) patients [69]. These studies demonstrate, for the first time, that serum and urine 2HG levels were markedly elevated in a IDH1-mutant breast adenocarcinoma patient, compared to the levels found in IDH1-wild-type patients (n= 6) [68]. They also reveal that serum 2HG levels were significantly elevated in IDH1/2-mutant (median 2.31 μM) versus the IDH1/2-wild-type (median 0.37 μM) ICC patients, suggesting that circulating 2HG may serve as a surrogate biomarker for IDH1/2 mutation status in ICC [69].

Reversed-phase chromatography is the chromatographic mode most suitable for coupling with ESI-MS because it typically uses aqueous-organic mobile phases containing

volatile buffers at low concentrations, which are ESI-compatible. However, conventional reversed-phase columns often do not provide adequate retention and separation of ionic compound, such as oncometabolites. Nevertheless, several reversed-phase-based LC-MS/MS approaches have been reported for the determination of oncometabolites in different biological samples [70–73], using reversed phase columns that, due to the design of its stationary phase, exhibit enhanced performance for the separation of polar compounds, such as the Acquity UPLC HSS T3 column with a trifunctional C18 alkyl phase bonded [70], the Kinetex core-shell C18 [71] and the Atlantis dC18 [72] columns, which are compatible with 100% aqueous mobile phases, and the Synergi Polar-RP column with an ether-linked phenyl phase [73].

A reversed-phase UHPLC-MS/MS method, using an Acquity UPLC HSS T3 C18 column, was applied to measure the levels of fumarate, succinate, and other TCA cycle metabolites, in tumour tissues samples from PHEOs/PGLs patients [70]. The method allowed to find that succinate was 25-fold higher in PPGLs with SDH mutations, whereas fumarate was 80% lower, revealing the role of the succinate/fumarate ratio at identifying and stratifying patients with SDH mutations [70]. Reversed-phase chromatography on a Kinetex-C-18 column, with 0.2% formic acid in water as mobile phase, was used to develop an UHPLC-MS/MS method for the simultaneous determination of fumarate, succinate, and other TCA cycle intermediates in different biological matrices [71]. The method was validated in human plasma, and applied only to the determination of citric acid in plasma samples from healthy people [71]. A reversed-phase UHPLC-MS/MS methodology, using an Atlantis dC18 column, was recently developed and validated to quantify TCA cycle metabolites, including fumarate and succinate, in human serum, kasumi-1 cells, and murine liver tissue samples [72]. Reversed-phase chromatography on a Synergi Polar-RP column, coupled with QqQ-MS/MS, was used to develop and validate a method that allows the simultaneous

determination of oncometabolites (succinate, fumarate and 2HG), and other TCA cycle metabolites (α-ketoglutarate, malic acid, and glutamate), in frozen and FFPE tissues from glioma patients [73]. The method was validated for quantitation of oncometabolites in pooled homogenates of tissues extracts [73]. Although oncometabolites were notably lost during the routine FFPE process, the ratios succinate/glutamate, fumarate/α-ketoglutarate, and 2HG/glutamate remained consistent between FFPE and matched frozen tissue specimens. Therefore, these ratios seem reliable surrogate measurements for the detection of altered levels of oncometabolites in FFPE specimens. The method was applied to the analysis of a collection of hereditary and sporadic renal cell carcinoma frozen tumour tissue samples, showing an abnormally high accumulation of fumarate and succinate in the specimens derived from Hereditary Leiomyomatosis and Renal Cell Cancer (HLRCC), and Hereditary Paraganglioma – Pheochromocytoma (PGL/PCC), respectively [73].

Ion pairing-reversed phase chromatography, using volatile amphiphilic amines as ion pair reagents in the aqueous-organic mobile phase, is a good alternative to reversed-phase chromatography for the separation of oncometabolites. An ion-paired reversed phase LC-MS/MS method, using ammonium formate as ion pair modifier, was applied to the simultaneous determination of 2HG and TCA cycle metabolites in glioma, primary glioblastomas, and nonglioma brain tumour tissue samples [74]. The study showed that, although 2HG levels accumulate in IDH mutated samples, they are not useful to identify malignant transformation, or to evaluate malignant progression [74]. Ion-paired with tributylammonium acetate (TBAA) was used to develop and validate a LC-MS/MS method for the quantification of 29 intracellular metabolites involved in TCA cycle, glycolysis and pentose phosphate pathway, in cell extracts of *E. coli*, finding that most of the targeted intracellular metabolites including fumarate and succinate are within the detection range [75]. This validated method was later adapted by different authors for the simultaneous

determination of 2HG, fumarate, succinate, and other TCA cycle metabolites in tumour tissues from glioma patients, showing for the first time, as already mentioned, that accumulation of 2HG is characteristic of gliomas harbouring IDH1 mutations [14]. Subsequently, the method was also applied to study the value of 2HG levels in serum, urine [76,77] and cerebrospinal fluid (CSF) [77] from glioma patients, as markers of IDH mutational status. Results of these works showed that the serum to urinary 2HG concentration ratio [76], or the urinary level of 2HG [77], could predict the presence of IDH1/2 mutations in gliomas. Similar studies were carried out in AML, using the same ion-pairing reversed-phase LC-MS/MS method, to determine 2HG in AML cells [78], serum [78–81], urine [79,80] and bone marrow aspirates [79–82], from AML patients. Results showed that serum 2HG levels were significantly higher in patients with IDH mutations (median 20.48 μM), compared with patients with IDH wild-type AML (median 0.41 μM) and healthy people (median 0.32 μM) [81]. It was also reported that the levels of 2HG in serum, urine and bone marrow aspirate are elevated in IDH-mutant patients, and that serum and urine 2HG levels decrease with response to therapy, in a consistent and predictable fashion [79]. All these results suggest that serum and/or urine 2HG levels may serve as non-invasive biomarkers of disease activity, IDH mutational status, and response to therapy in patients with AML [79,81].

The majority of these publications, presented in Table 3, aims to explore the clinical usefulness of oncometabolites as biomarkers in different type of cancers [68,69,82,70,74,76–81]. Therefore, oncometabolite determination is carried out using LC-MS methods previously reported in the literature for the determination of other metabolites, and the authors do not provide information about their validation parameters. There are only four publications focused on the development and validation of LC-MS methodologies for the quantification of oncometabolites in biological samples [71–73,75]. The main analytical characteristics, in terms of LODs and LOQs, intra and inter-day precision and recovery, of

these validated LC-MS methods are presented in Table 4. These developed and validated LC-MS/MS methodologies [71–73,75] allow the quantification of oncometabolites in biological samples (serum, plasma, tissues and cultured cells) at the low μM level; e.g. LOQs in tissues of 0.0020 μM for 2HG [73], 0.2 μM for fumarate [72,73] and in the range of 0.02-0.198 for succinate [72,73]. Low LOQs are also obtained for succinate and fumarate in plasma (0.18 and 3.0 μM) [71] serum (0.198 and 0.202 μM) [72] and cultured cells (0.102 and 0.0388 μM) [75]. The Intra- and inter-day precision of these methods for individual oncometabolites is within the generally accepted criteria for bioanalytical method validation (below 20%), with satisfactory quantitative recoveries. Compared to GC-MS/MS methods (Table 2), these LC-MS/MS methods show LODs and LOQs that are in the same order of magnitude, or about one order of magnitude higher, than those reported for GC-MS methods, depending on the oncometabolite and the sample. However, the LC-MS methods do not need a derivatization stage prior to separation.

Determination of small carboxylic acids, such as oncometabolites, by LC-MS- based methodologies is carried out under negative ESI mode (Table 3). However, the detection sensitivity in this mode is often problematic, due to reduced ionization efficiency and increased ion suppression [83]. Therefore, to improve the ESI-MS response, several derivatization reagents suitable for the positive ion detection of the derivatized species have been developed [83], such as N-methyl-2-phenylethanamine (MPEA) [84], O-benzylhydroxylamine (O-BHA) [85], 4-bromo-N-methylbenzylamine (4-BNMA) [86] and 4-[2-(N,N-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxa-diazole (DAABD-AE) [87]. These reagents selectively react with carboxylic acids, forming derivatives that are suitable for separation by reversed phase LC, in combination with ESI-MS detection under sensitive positive ESI mode, ESI(+).

To date, several reversed phase LC-ESI(+)-MS methods, based on derivatization strategies with the above mentioned reagents, have been reported for the determination of oncometabolites in different biological samples, including cultured cells [85,86], tumour xenografts [85], tissues [84,86], and dried blood spots (DBS) [87]. These developed methods are summarized in Table 5. Typical sample preparation procedures, such as metabolism quenching together with protein precipitation with ice-cold perchloric acid [84], or ice-cold 80% (v/v) methanol/water [85], and only protein precipitation with 50% (v/v) methanol/water [86], are carried out prior to oncometabolite derivatization. Additional on-line solid-phase extraction (SPE) clean-up, using a C8 cartridge (4 mm × 2 mm, 5 μm), has also been described [84]. In the case of DBS samples, extraction and derivatization of targeted metabolites were achieved in a single step by incubating the DBS sample directly with the derivatization reagents [87]. Targeted quantitative analysis is mainly performed via MRM mode on QqQ-MS/MS instruments, using the internal external calibration method with isotopically labelled (e.g. fumarate-^{13}C4, succinate-d4, 2HG-d4, succinate-d6, 2HG-d3, citric acid-d4) internal standard [85–87], to correct for any possible errors, along the steps of the analytical procedure, that can affect the obtained signal.

Derivatization with MPEA has been used to develop a reversed-phase LC-ESI(+)-TOF-MS method, able to determine several TCA cycle metabolites, including fumarate and succinate, in biological samples [84]. The applicability of the developed method in metabolomic studies was illustrated by analyzing heart tissue samples, with and without previous ^{13}C-acetate labelling, in order to enable ^{13}C-flux analysis. In another approach, O-BHA was used as derivatization reagent to simultaneous quantify 2HG and all common TCA cycle metabolites by reversed-phase LC–ESI(+)-QqQ-MS/MS [85]. The method was validated for quantitation of all targeted analytes in cultured breast cancer cells and tumour xenografts, and it was successfully applied to quantify changes in the levels of lactate and α-

ketoglutarate in these samples, after treatment with a drug that disrupted the TCA cycle [85]. An alternative derivatization strategy for the determination of several biologically important carboxylic acids, including 2HG, fumaric and succinic acids, by reversed-phase LC-ESI(+)-QqQ-MS/MS, has been proposed, using 4-BNMA as derivatization reagent, which incorporates a bromine in the derivatized product, allowing for improved MS identification due to the isotope pattern of bromine [86]. This method was validated and successfully applied to the analysis of model biological samples (porcine skeletal muscle, heart tissue, and cardiac and cancer cultured cells). Finally, derivatization with DAABD-AE was used to develop a reversed-phase LC-ESI(+)-QqQ-MS/MS method, to determine some TCA cycle metabolites (citric, 2-ketoglutaric, succinic, fumaric, and malic acid) in dried blood spots from healthy and diseased individuals (patients with propionic aciduria and methylmalonic aciduria) [87].

Table 5 shows some of the main analytical characteristics, such as LOQs, inter-day precision and recovery, that have been reported for these developed methods. These data show that, although the derivatization process introduce an additional working step, making the analysis longer and more complex, the derivatization with O-BHA and 4-BNMA seems to improve the LOQs for the determination of oncometabolites in cultured cells and tissues samples, in comparison with the LOQs obtained by the LC-MS methods without derivatization (see Table 4). However, the LOQs do not improve when the derivatization is carried out with MPEA or DAABD-AE reagents (see Table 2).

4.3. GC-MS-based and LC-MS-based methodologies for determination of 2HG enantiomers

All the analytical methods summarized in Tables 1, 3 and 5, when applied to the determination of 2HG, are not able to differentiate between D-2HG and L-2HG and,

consequently, the sum of the two enantiomers is measured. However, the oncometabolite formed by mutations affecting IDH1/2 genes was demonstrated to be D-2HG [14,17,18]. Therefore, determination of D-2HG and L-2HG, instead of total 2HG, should be performed in clinical and scientific research.

Various GC-MS approaches have been developed to measure 2HG enantiomers, either indirectly, using chiral derivatization reagents [88–93], or directly, by means of chiral columns [94]. Indirect GC-MS methods were initially developed to analyse urine samples of organic aciduria patients for the differential diagnosis of 2HG acidurias [16]. In these methods, 2HG was extracted with ethyl acetate and, after drying, its diastereoisomers were created by esterification of the carboxyl groups with D-2-butanol, with subsequent acylation of the remaining hydroxyl group (see structure of 2HG in Fig. 1); in this way, D-2HG and L-2HG can be measured, separately, as O-acetylated di-(D)-2-butyl ester derivatives, using achiral capillary GC columns [88–91]. These methods were later applied by other authors, to determine 2HG enantiomers in colorectal cancer cells to explore their role in this type of cancer [92]; and in serum from AML patients, to test their diagnostic value to identify IDH1/2 mutations [93]. The analysis was performed by GC-QqQ-MS/MS, in selected-reaction monitoring (SRM) mode, using 2HG-^{13}C4 as the internal standard [93]. The results obtained show that, in patients with AML with IDH1/2 mutations, the D-2HG to L-2HG concentration ratios in serum were significantly higher (range, 3.1 to 51.2) than those in patients with IDH1/2 wild type (range, 0.2 to 1.9). Thus, the authors conclude that the D to L ratio is an adequate predictor of both the presence of IDH1/2 mutations and of disease outcome in AML patients [93]. These indirect GC-MS methods for the determination of 2HG enantiomers have not been fully validated, and they have the disadvantage of being time-consuming, due to the two-step chiral derivatization procedure. To avoid this chiral derivatization step, a chiral GC capillary column, CP-Chirasil-Dex CB, was used, in a recently published study, to

develop and validate a GC-QqQ-MS/MS methodology to determine 2HG enantiomers in serum [94]. The enantiomers were extracted from the sample with ethyl acetate and converted into volatile methyl esters before analysis. Quantification was performed in multiple reaction monitoring (MRM) mode, using deuterated 2HG as internal standard. The validated method presents low quantification limits (0.071 μM for both enantiomers) and good inter-day precision (RSD of 9.8%), but the recovery ranged from 15.6 to 23.0% for D-2HG, and from 11.1 to 21.2% for L-2HG [94]. The method was applied to establish reference values for healthy physiological levels of 2HG enantiomers, showing an average serum concentration of total 2HG (sum of the two enantiomers) of 2.20 μM (n=60), and an average serum D-2HG/L-2HG ratio of 1:1. The feasibility of this method for clinical use was established in a pilot study, with serum from patients with and without IDH mutant gliomas, that clearly showed the necessity of determining both enantiomers separately, because neither the total 2HG levels, nor the D-2HG/L-2HG ratio, seemed to provide accurate enough patient information [94].

Several LC-MS/MS methodologies, which are summarized in Table 6, have been also reported for the determination of 2HG enantiomers in biological samples, by using chiral derivatization reagents, such as diacetyl-L-tartaric anhydride (DATAN) [95–100] and N-(p-toluenesulfonyl)-L-phenylalanyl chloride (TSPC) [73,101]. These derivatization reagents react with the hydroxyl group on the asymmetric carbon of 2HG enantiomers (see Fig. 1) to form diastereoisomers, which are separated by ion-paired reversed-phase LC [95–100], or by reversed-phase LC [73,101], on conventional C18 columns coupled mostly to ESI(-)-QqQ-MS/MS. Quantification is carried out in MRM mode using mostly the isotope-labelled (e.g. D,L-2HG-^{2}H4, D,L-2-hydroxyglutaric acid-d4, or D,L-2HG-d3) internal standard calibration method [73,95–100]. Quantification by the standard addition method has also been reported [101]. Prior to chiral derivatization, sample preparation was performed differently, depending

on the type of biological sample. Thus, urine, samples were diluted with methanol [95], or centrifugated and supernatant filtered [101]; and CSF samples were ultrafiltered with 10 kDa membrane filters [98]. In the case of serum samples, the targeted analytes were extracted with methanol [97], or by using a solid phase extraction anion exchange and reversed phase STRATA^{TM}XL-A (200 mg-3mL) cartridge [99,100]. Finally, tissue samples were extracted with 80% (v/v) methanol/water, either precooled on dry ice [96], or pre-chilled at -80 ºC [101], or with ice-cold methanol followed by 80% (v/v) methanol/water [73].

Chiral derivatization with DATAN was initially used to develop and validate a LC-MS/MS method for the determination of 2HG enantiomers in human urine [95], as a good alternative to the already mentioned GC-MS methods used in clinical laboratories for the differential diagnosis of 2HG acidurias [88–91]. This validated LC-MS/MS methodology was later adapted by other researchers to quantify the levels of 2HG enantiomers in papillary thyroid carcinoma (PTC) tissues, showing accumulation of both enantiomers in these tumours, without IDH1/2 mutations [96]; and, in serum from patients with myelodysplastic syndromes (MDS), showing that 2HG levels were elevated significantly more often in MDS patients with IDH mutations than with IDH wild type [97]. LC-MS/MS methodologies based on chiral derivatization with DATAN have also been developed and validated to determine the levels of 2HG enantiomers in CSF samples from glioma patients [98], and in serum from AML patients [99], to test their relevance as biomarkers of IDH mutational status. In a similar study, this last methodology was later applied, by other authors, to analyse serum from intrahepatic cholangiocarcinomas (ICC) patients [100]. The results of these studies showed that D-2HG levels in biological fluids, such as CSF and serum, allow to predict and monitor the presence of IDH mutations in glioma [98], AML [99] and ICC [100] cancers.

As shown in Table 6, 2HG enantiomers in biological fluids can be quantified at the low μM level by these LC-MS/MS methodologies, using DATAN as chiral derivatization reagent,

with LOQs of 1μM in urine [95], 0.1 μM in CSF [98] and 0.2 μM in serum [99]. The precision is good, with inter-day RSD% below 18% [95,98,99]. Recoveries in urine and CSF were in the range of 91-109% [95,98], but in serum were only 31%, because in this case the methodology involved a sample extraction procedure using SPE on an STRATA^{TM}XL-A cartridge [99].

Chiral derivatization with TSPC has been proposed as an advantageous alternative to DATAN, for the LC-MS/MS determination of 2HG enantiomers [73,101] because this approach can improve the chromatographic separation of the derivatized enantiomers under reversed-phase LC and also the MS detection sensitivity (101). TSPC was used as derivatization reagent to develop and validate a LC-ESI(-)-MS/MS method to determine 2HG enantiomers in urine from healthy individuals and patients with type 2 diabetes mellitus, lung cancer, colorectal cancer and nasopharyngeal carcinoma, finding that there are no significant differences in the contents of 2HG between patients and healthy controls. The method was also applied to the analysis of human clear cell renal cell carcinoma (ccRCC) tissues, showing large increases of both 2HG enantiomers in ccRCC tissues, compared to normal ones [101]. In this work, the authors have also used DATAN for the derivatization of 2HG enantiomers to compare with the TSPC reagent. They found that the resolution of the separation of DATAN derivatives was similar as that of TSPC derivatives, but the LODs of D-2HG and L-2HG upon TSPC derivatization were 0.00012 and 0.00010 μM, respectively, which are about 100 folds lower than those obtained with DATAN e.g. 0.0115 and 0.010 μM, respectively [101]. This means that the estimate LOQs for both enantiomers is around 0.0004 μM which is much lower than those obtained by other authors using DATAN as derivatization reagent (Table 6). Good precision was also achieved with inter-day RSD less than 10.7% and 12.1% for D-2HG and L-2HG, respectively, and recoveries in urine samples in the range of 88.0-110.9 %. Recently, TSPC was also used to develop and validate a LC-MS/MS

method to determine D- and L-2HG in frozen and FFPE glioma tissue samples, finding that D-2HG levels, but not L-2HG, were significantly increased in the IDH1-mutated glioma samples, as compared to the IDH wild-type samples [73]. Validation analytical parameters for this methodology, presented in Table 6, shows LOQs for both enantiomers of 0.002 μM [73], which are considerably lower than those obtained with DATAN derivatization (Table 6). Good precision was also achieved with inter-day RSD% lower than 5.5%. Finally, quantitative recoveries were obtained from pooled homogenate of FFPE or frozen tissue [73].

5. Conclusions

There is now increasing evidence that in tumours associated with dysfunction of the SDH, FH, and IDH enzymes the underlying mechanisms of tumorigenesis involve the aberrant accumulation of the oncometabolites succinate, fumarate, and 2HG. Therefore, the levels of these oncometabolites in biological fluids, cancer cells and tumour tissues have been proposed as potential clinically useful tumour-specific biomarkers.

To date, many analytical strategies based on LC-MS and GC-MS techniques have been applied to determine oncometabolites in different samples of biological origin, mainly cultured cancer cell lines, and biofluids (serum, urine, CSF) and tumour tissues from patients with different types of oncometabolite related cancers (AML, glioma, PHEOs/PGLs). However, most of them focus on the targeted determination of only 2HG levels, including total 2HG, and separate D- and L-2HG enantiomers, while the other two oncometabolites are usually determined together with other TCA cycle intermediates. Moreover, only a very few number of the reported GC-MS and LC-MS methods enable the simultaneous determination of the three oncometabolites (fumarate, succinate and 2HG).

The oldest a most common analytical approach for the quantification of oncometabolites in biological samples is the GC-MS based method, which is commonly used

in clinical laboratories for urine organic acid analysis. However, very few GC-MS methods have been specifically developed and validated for the determination of oncometabolites in biological samples. The developed methodologies provide enough sensitivity for the determination of oncometabolites in the low μM range, which is low enough to determine oncometabolites in biological samples, but they are time consuming due to the derivatization step. Therefore, other analytical strategies based on LC-MS/MS have been proposed as a good alternative to the GC-MS methods for the determination of oncometabolites. Although most of them are not fully validated for this type of analysis, the validated methodologies show similar or lower sensitivity in comparison with GC-MS methods. A significant strategy to improve the sensitivity of the analysis of oncometabolites by LC-MS/MS is the use of chemical derivatization with reagents such as O-BHA and 4-BNMA, but as in the case of GC-MS, the derivatization process introduces an additional working step, making the analysis longer and more complex.

In conclusion, further studies are still required to develop reliable and clinically feasible GC-MS and LC-MS methodologies, fully validated, for the determination of oncometabolites in biological samples for clinical and translational research purposes. Moreover, larger prospective clinical studies, with large cohorts, are also necessary to establish the clinical utility of oncometabolites.

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Figure 1

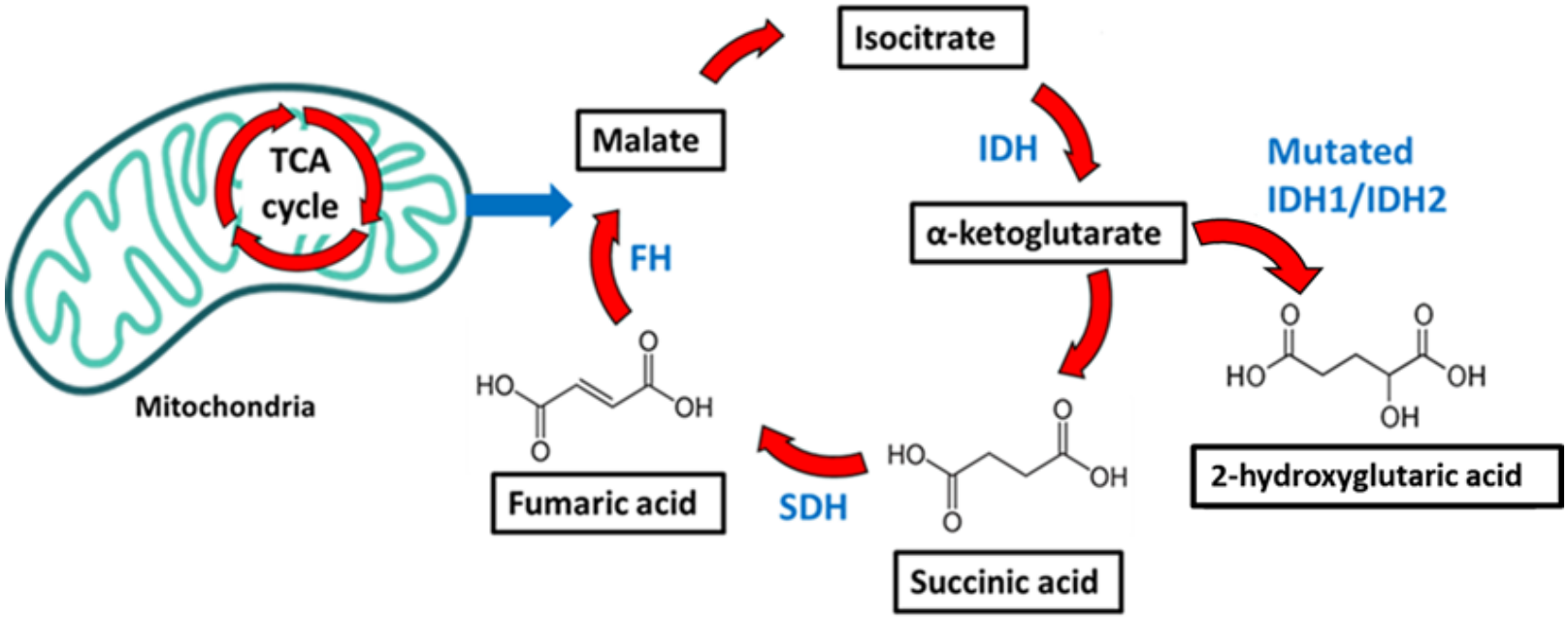

Figure 1. Formation and chemical structure of oncometabolites. FH: Fumarate hydratase; SDH: Succinate dehydrogenase; IDH: Isocitrate dehydrogenase.

Figure 2

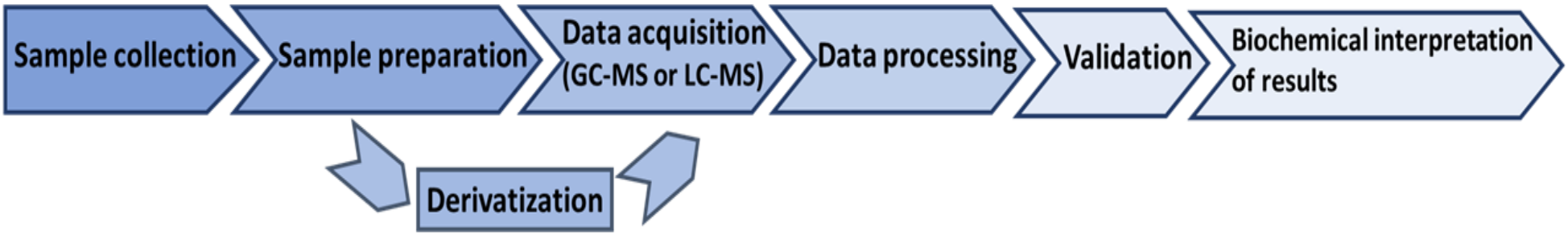

Figure 2. Typical chromatographic-MS based targeted metabolomic workflow

Legends of the figures

Figure 1. Formation and chemical structure of oncometabolites. FH: Fumarate hydratase; SDH: Succinate dehydrogenase; IDH: Isocitrate dehydrogenase.

Figure 2. Typical chromatographic-MS based targeted metabolomic workflow

Tables

Table 1. Summary of GC-MS methods for targeted determination of oncometabolites in biological samples

Analyte	Sample	Sample preparation	Derivatization	Column	Detection; quantification mode	Reference
2HG	Serum from AML patients	No reported	MSHFBA	DB-5MS (30 m x 0.25 mm; film thickness: 0.25 µm)	EI-qMS, SIM mode	[47]
2HG	Formalin-fixed paraffin-embedded glioma tissues	Deparaffinization with xylene; homogenization by sonication with water; acidification with 5M HCl; addition of IS (3-hydroxygluratic acid-d4) and solid NaCl; Extraction twice with ethyl acetate; organic layer evaporation to dryness; derivatization	MSHFBA	DB-5MS (30 m x 0.25 mm; film thickness: 0.25 µm)	EI-qMS, SIM mode; isotope-labelled internal standard calibration	[48]
2HG	Serum from glioma patients	No reported	MSHFBA	DB-5MS (30 m x 0.25 mm; film thickness: 0.25 µm)	EI-qMS, SIM mode	[49]
2HG	Serum from AML patients	Acidification with 5M HCl; addition of IS (3-hydroxygluratic acid-d4) and solid NaCl; extraction twice with ethyl acetate; organic solvent organic layer evaporation to dryness; derivatization	MSHFBA	DB-5MS (30 m x 0.25 mm; film thickness: 0.25 µm)	EI-qMS, SIM mode; isotope-labelled internal standard calibration	[50]
2HG	Wild-type and mutant IDH cancer cell lines	Extraction with 80% (v/v) methanol/water at -80 ºC; centrifugation; supernatant clean-up by elution from an AG-1 XB 100-200 anion exchange resin in 3N HCl; eluate	MTBSTFA	HP-5MS (30 m x 0.25 mm; film thickness: 0.25 µm)	EI-qM, Full Scan mode; external standards calibration	[51-53]

		evaporation to dryness; derivatization				
2HG	Mutant IDH cancer cell lines	Extraction with 80% (v/v) methanol/water at -80 ºC containing the IS (heptadecanoic acid); centrifugation; supernatant evaporation to dryness; derivatization	MTBSTFA	HP-5MS (30 m x 0.25 mm; film thickness: 0.25 µm)	EI-qMS, Full Scan mode; internal standard calibration	[54]
2HG	Glioma tissues	Extraction with 80% (v/v) methanol/water at -80 ºC; centrifugation; supernatant evaporation to dryness; derivatization	MTBSTFA	HP-5ms Intuvo (30 m x 0.25 mm; film thickness: 0.25 µm)	EI-qMS, Full Scan mode; external standards calibration	[55]
Fumarate, succinate and other energy metabolites	Liver tissue and serum from obese mice	Tissue: addition of IS (D,L-norleucine); homogenization and extraction with 70% (v/v) acetonitrile/water using zirconia/silica beads; centrifugation; supernatant evaporation to dryness; derivatization Serum: addition of IS (D,L-norleucine); extraction with acetonitrile; centrifugation; supernatant evaporation to dryness; derivatization	MTBSTFA + 1% TBDMCS	HP-5MS (30 m x 0.25 mm; film thickness: 0.25 µm)	EI-qMS, SIM mode; internal standard calibration	[59]
Fumarate, succinate and other TCA cycle metabolites	Normal and cancerous tissues from gastric adeno-carcinoma patients	Homogenization with an Ultra-Turrax® disperse; extraction with 50% (v/v) acetonitrile/water containing the IS(3,4-dimethoxybenzoic acid); centrifugation; supernatant collection; alkalinisation to pH >12 with 5.0M NaOH; derivatization of carbonyl groups with methoxyamine hydrochloride; acidification to pH 1-2 with 10%	MTBSTFA + 1% TBDMCS	HP Ultra-2 (25 m × 0.20 mm; film thickness: 0.11 µm)	EI-qMS, SIM mode; internal standard calibration	[60]

		sulphuric acid solution saturated with sodium chloride; extraction with diethyl ether followed by ethyl acetate; addition of trimethylamine; evaporation of combined extracts to dryness; derivatization.				
Fumarate and Succinate	Tumour tissue from PHEOs/PGLs patients	Extraction with perchloric acid; centrifugation; neutralization of supernatant; addition of IS (fumaric acid-^{13}C4, succinic acid-^{13}C4); evaporation to dryness; derivatization	MTBSTFA + 1% TBDMCS	DB-1 (30 m × 0.25 mm)	EI-qMS, SIM mode; isotope-labelled internal standard calibration	[61]
Fumarate, succinate and other TCA cycle and glycolysis metabolites	Cerebrospinal fluid from glioma patients	Centrifugation; supernatant extraction with methanol/water/ chloroform (2.5:1:1) containing the IS (2-isopropylmalic acid); centrifugation; supernatant lyophilization; derivatization	MSTFA	DB-5 (30 m x 0.25 mm; film thickness: 1.00 µm)	EI-qMS, Full Scan mode; internal standard calibration	[56]
2HG	Breast carcinoma cell lines	Extraction of pelleted cells with chloroform:methanol (2:1); centrifugation; evaporation of the upper polar phase; derivatization	MSTFA	No reported	EI-qMS, SIM mode; external standards calibration	[57]
2HG	Tumour tissue from breast cancer patients	Fresh-frozen tissue; extraction with aqueous methanol; centrifugation; supernatant collection; addition of IS (myristic acid-d27); evaporation to dryness; derivatization	BSTFA	No reported	EI-QqQ-MS/MS, SRM mode; internal standard calibration	[62]
2HG	Normal and cancerous tissue from head and neck squamous cell carcinoma patients	Homogenization with stainless steel beads and sonication; centrifugation; supernatant extraction with methanol; centrifugation;	BSTFA	No reported	EI-qMS, SIM mode; external standards calibration	[58]

		supernatant evaporation to dryness; derivatization				
Fumarate, succinate and other TCA cycle metabolites	Serum from patients with cardiovascular diseases	Addition of IS (glutamic acid-^{15}N); extraction with methanol at −20 °C; centrifugation; supernatant clean-up with chloroform; evaporation of the upper polar phase; derivatization	BSTFA + 2% TMCS	VF-5 ms (30 m × 0.25 mm; film thickness: 0.25 µm)	EI- ion trap- MS, SIM mode; internal standard calibration	[63]
2HG	Serum from healthy individuals and AML patients	Addition of IS (L-2-chlorophenylalanine, heptadecanoic); extraction with methanol/chloroform (3:1); centrifugation; supernatant evaporation to dryness; derivatization	BSTFA + 1% TMCS	B-5MS (30 m × 0.25 mm; film thickness: 0.25 µm)	EI-TOF-MS, Full Scan mode; internal standard calibration	[64]
2HG	Human serum	Addition of IS (2HG-^{13}C5) acidification with 5M HCl; extraction with ethyl acetate; centrifugation; organic phase evaporation to dryness; derivatization under microwave irradiation to reduce the derivatization time	BSTFA + 1% TMCS	Sapines-5MS+ (30 m × 0.25 mm; film thickness: 0.25 µm)	EI-qMS, SIM mode; isotope-labelled internal standard calibration	[65]
2HG, fumarate, succinate and other TCA cycle metabolites	Serum from nasopharyngeal carcinoma patients	Addition of IS (2-isopropylmalic acid); extraction with 95% (v/v) methanol/water; centrifugation; supernatant evaporation to dryness; derivatization	BSTFA + 1% TMCS	HP-5 MS (30 m × 0.25 mm; film thickness: 0.25 µm)	EI-qMS, SIM mode; internal standard calibration	[66]
Fumarate, succinate and other TCA cycle and glycolysis metabolites	Hepatic cell lines	Addition of IS (myristic acid- ^{13}C2) to the cells; quenching with 60% (v/v) aqueous methanol buffered with 70 mM of HEPES at −50 °C; extraction with 75% (v/v) aqueous methanol/methyl tert-butyl ether (9:1); three cyles of freeze-thaw (liquid nitrogen and 37ºC water bath);centrifugation;	BSTFA + 1% TMCS	TG-5MS (30 m × 0.25 mm; film thickness: 0.25 µm)	EI-QqQ-MS/MS, SRM mode; internal standard calibration	[67]

		supernatant evaporation to dryness; derivatization				

Abbreviations**:** AML: Acute myeloid leukaemia; 2HG: 2-hydroxyglutarate; BSTFA: N,O-bis-(trimethylsilyl)trifluoroacetamide; EI- ion trap- MS: Electron impact ion trap mass spectrometry; EI-qMS: Electron impact quadrupole mass spectrometry; EI-QqQ-MS/MS: Electron Impact triple quadrupole tandem mass spectrometry; EI-TOF-MS: Electron impact time of flight mass spectrometry; HEPES: 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane sulfonic acid; IDH: Isocitrate dehydrogenase; IS: Internal standard; MSHFBA: N-methyl-N-(trimethylsilyl)heptafluorobutyramide; MSTFA: N-methyl-N-(trimethylsilyl)trifluoroacetamide; MTBSTFA: N-methyl-N-(tertbutyldimethylsilyl)trifluoroacetamide; PHEOs/PGLs: Pheochromocytomas/ paragangliomas; SIM: Selected ion monitoring; SRM: Selected reaction monitoring; TBDMCS: tert-butyldimethylchlorosilane; TCA: Tricarboxylic acid; TMCS: trimethylchlorosilane.

Table 2. Summary of performance characteristics among validated GC-MS methods for oncometabolite quantification in biological simples.

Sample	Oncometabolite	LOD (μM)	LOQ (μM)	[a]Precision (% RSD)		Recovery (%)	Reference
				Intra-day	Inter-day		
Human serum	2HG	No reported	0.0675	2.1-7.2	2.4-19.5	99.5-105.5	[65]
Human serum	Succinate	0.0169	0.0847	4.3-5.9	5.8-7.2	93.3-105.1	[66]
	Fumarate	0.0172	0.0862	4.4-7.2	5.4-7.6	92.9-103.9	
	2HG	0.0675	0.2430	5.6-7.4	6.7-9.0	89.6-92.8	
Human serum	Succinate	0.0169	0.0565	5.3	15.8	99.8	[63]
	Fumarate	0.0172	0.0575	5.2	5.8	100.1	
Mice liver tissue and serum	Succinate	0.01	0.5	2.0-10.2	5.06-8.75	No reported	[59]
	Fumarate	0.03	0.5	2.58-8.46	3.85-7.17		
Cultured cells	Succinate	0.661	1.32	3.57-13.9	9.63-14.8	87.1-106	[67]
	Fumarate	0.0422	0.168	5.83-17.7	10.8-16.4	102-112	

Abbreviations: 2HG: 2-hydroxyglutarate; LOD: limit of detection; LOQ: limit of quantification

[a]Precision expressed as percent of relative standard deviation (% RSD)

Table 3. Summary of LC-MS methods for the determination of oncometabolites in biological samples

Analytes	Sample	Sample preparation	Separation mechanism	Separation conditions (column; mobile phase)	Detection; quantification mode	Reference
2HG	Serum and urine from breast adenocarcinoma patients	Extraction with methanol containing the IS (2HG-^{13}C5); centrifugation; supernatant evaporation to dryness; reconstitution in 0,1% formic acid in water	Ion-exchange	Bio-Rad Fast Acid Analysis column (7.8 x 100 mm, 9 µm); 0.1 % formic acid in water at a flow rate of 1mL min^{-1} in the isocratic elution mode	ESI(-)-QqQ-MS/MS, MRM mode; isotope-labelled internal standard calibration	[68]
2HG	Serum from patients with intrahepatic cholangiocarcinoma	Addition of IS (2HG-^{13}C5); extraction with acetonitrile; centrifugation; supernatant collection; dilution (1:1) with water	Ion-exchange	Bio-Rad Fast Acid Analysis column (7.8 X 100 mm, 9 µm); 0.1 % formic acid in water at a flow rate of 1mL min^{-1} in the isocratic elution mode	ESI(-)-QqQ-MS/MS, MRM mode; isotope-labelled internal standard calibration	[69]
Fumarate, succinate and other TCA cycle metabolites	Frozen and Formalin-Fixed Paraffin-Embedded tumour tissue from PHEOs/PGLs patients	Frozen tissue: extraction and homogenization with methanol containing the ISs (succinate-^{13}C4, fumarate-^{13}C4) by vortexing with a metal bead; centrifugation; supernatant evaporation to dryness; reconstitution in 0,2% formic acid in water Formalin-fixed paraffin-embedded tissue: extraction and homogenization with 80%(v/v) methanol/water containing the ISs (succinate- ^{13}C4, fumarate-^{13}C4) at 70°C to melt the paraffin; centrifugation;	Reversed - phase	Waters Acquity UPLC® HSS T3 column (2.1 × 100 mm,1.8 µm); 0.2% formic acid in water/ 0.2% formic acid in acetonitrile at a flow rate of 0.459 mL min^{-1} in the gradient elution mode.	ESI(-)-QqQ-MS/MS, MRM mode; isotope-labelled internal standard calibration	[70]

		supernatant evaporation to dryness; reconstitution in 0,2% formic acid in water				
Fumarate, succinate and other TCA cycle metabolites	Human plasma from healthy people	Addition of IS (citric acid-d4); extraction with 5% trichloroacetic acid; centrifugation; supernatant collection.	Reversed-phase	Kinetex-C18 column (2.1 x 100 m, 1.7 µm); 0.2 % formic acid in water at a flow rate of 0.4 mL min^{-1} in the isocratic elution mode.	ESI(-)-QqQ-MS/MS, MRM mode; matrix match calibration with internal standard.	[71]
Fumarate, succinate and other TCA cycle metabolites	Human serum, Kasumi-1 cell line and mouse liver tissue	Serum: addition of ISs(succinate-$^{13}C4$, fumarate-$^{13}C4$); extraction with1% formic acid in acetonitrile; filtration; filtrate collection Cells: addition of ISs(succinate-$^{13}C4$, fumarate-$^{13}C4$) and mix with 0.1% formic acid in water; extraction with1% formic acid in acetonitrile; sonication; centrifugation; supernatant filtration; filtrate collection Frozen tissue: addition of ISs (succinate-$^{13}C4$, fumarate-$^{13}C4$); extraction and homogenization twice with 1% formic acid in acetonitrile and 1% formic acid in 90% (v/v) acetonitrile/water; centrifugation; supernatants filtration; filtrate collection	Reversed-phase	Atlantis dC18 column (2.0 x 100 mm, 3 µm) 0.1% formic acid in water/ acetonitrile at a flow rate of 0.6 mL min^{-1} in the gradient elution mode	ESI(-)-QqQ-MS/MS, MRM mode; isotope-labelled internal standard calibration	[72]
2HG, fumarate, succinate and other TCA	Frozen and Formalin-Fixed Paraffin-Embedded	Deparaffinization of FFPE tissue with xylene; homogenization of	Reversed-phase	Phenomenex Synergi^{TM} Polar-RP column (2 x150 mm, 4 µm); 0.03% formic	ESI(-)-QqQ-MS/MS, MRM mode; isotope-labelled internal	[73]

cycle metabolites	tumour tissues from glioma patients	deparaffinized or frozen tissue in water using a Precellys® homogenizer; addition of ISs (succinate-d6, fumarate-^{13}C2-d2, 2HG-d3); extraction twice with ice-cold methanol and 80% (v/v) methanol/water; centrifugation; supernatants evaporation to dryness; reconstitution in water		acid in water/ 0.03% formic acid in acetonitrile at a flow rate of 0.25 mL min^{-1} in the gradient elution mode	standard calibration	
2HG, fumarate, succinate and other TCA cycle metabolites	Normal brain tissue and tumour tissue from glioma and primary glioblastoma patients	Addition of ISs (succinate-^{13}C4, fumarate-^{13}C4); homogenization and extraction with cold methanol using a SilentCrusher M; centrifugation; supernatant evaporation to dryness; reconstitution in initial mobile phase	Ion pairing-reversed phase	Agilent ZORBAX SB-Aq column (3.0 x 150 mm, 3.5 µm); 5 mM ammonium formate (pH 2 adjusted with formic acid)/ acetonitrile at a flow rate of 0.3 mL min^{-1} in the gradient elution mode	ESI(-)-QqQ-MS/MS, MRM mode; isotope-labelled internal standard calibration	[74]
Fumarate, succinate and other 27 metabolites involved in TCA cycle, glycolysis and pentose phosphate pathway	Cell extracts from *Escherichia coli*	Quenching with 60%(v/v) methanol/water at -50ºC; centrifugation; cell pellet resuspension by vortexing with 60% (v/v) methanol/water at −20ºC; extraction with 0.3M KOH dissolved in 25% ethanol; neutralization with acetic acid; centrifugation; supernatant collection	Ion pairing-reversed phase	Synergi Hydro-RP colum (2.1mm x 150 mm, 4µm); 10 mM tributylamine with 15 mM acetic acid (pH 4.95)/ methanol at a flow rate of 0.2 mL min^{-1} in the gradient elution mode	ESI(-)-QqQ-MS/MS, SRM mode; standard addition calibration	[75]

2HG, fumarate, succinate and other TCA cycle metabolites	Tumour tissue from glioma patients	Extraction with 80% (v/v) methanol/water at -80 °C followed by homogenization at 4 °C; centrifugation; supernatant collection	Ion pairing-reversed phase	Synergi Hydro-RP colum (2 x 150 mm, 4 µm); 10 mM tributylamine with 10 mM acetic acid (pH 5.5)/ methanol at a flow rate of 0.2 mL min^{-1} in the gradient elution mode	ESI(-)-QqQ-MS/MS, MRM mode; external standards calibration	[14]
2HG	Serum and urine from glioma patients	Serum: addition of IS (pentanoic acid-d4); extraction with acetonitrile; centrifugation; supernatant evaporation to dryness; reconstitution in initial mobile phase Urine: 40-fold dilution in initial mobile phase containing the IS (pentanoic acid-d4);	Ion pairing-reversed phase	Synergi Hydro-RP colum (2.1 x 150 mm, 4 µm); 10 mM tributylamine with 15 mM acetic acid (pH 4.6)/ methanol at a flow rate of 0,3 mL min^{-1} in the gradient elution mode	ESI(-)-QqQ-MS/MS, MRM mode; isotope-labelled internal standard calibration	[76]
2HG	Serum, urine and cerebrospinal fluid from glioma patients	Extraction with 80% (v/v) methanol /water at -80°C; centrifugation; supernatant evaporation to dryness; reconstitution in initial mobile phase	Ion pairing-reversed phase	Synergi Hydro-RP colum (2.1 x 150 mm, 4µm); 10 mM tributylamine with 15 mM acetic acid (pH 4.95)/ methanol at a flow rate of 0,2 mL min^{-1} in the gradient elution mode	ESI(-)-QqQ-MS/MS, MRM mode; external standards calibration	[77]
2HG	Serum from AML patients and AML cell lines	Extraction with 80% (v/v) methanol /water at -80°C; centrifugation; supernatant evaporation to dryness; reconstitution in initial mobile phase .	Ion pairing-reversed phase	Synergi Hydro-RP colum (2 mm x 100 mm, 2.1 µm); 10 mM tributylamine with 10 mM acetic acid (pH 5.5)/ methanol at a flow rate of 0,2 mL min^{-1} in the gradient elution mode	ESI(-)-QqQ-MS/MS, MRM mode; external standards calibration	[78]
2HG	Serum, urine and bone marrow aspirates from AML patients	Extraction with 80% (v/v) methanol /water at -80°C; centrifugation; supernatant evaporation to	Ion pairing-reversed phase	Synergi Hydro-RP colum (2 mm x 100 mm, 2.1 µm); 10 mM tributylamine with 10 mM acetic acid (pH 5.5)/ methanol at a	ESI(-)-QqQ-MS/MS, MRM mode; external standards calibration	[79,80]

		dryness; reconstitution in initial mobile phase		flow rate of 0,2 mL min^{-1} in the gradient elution mode		
2HG	Serum from AML patients	Extraction with 80% (v/v) methanol /water at -80°C; centrifugation; supernatant evaporation to dryness; reconstitution in initial mobile phase	Ion pairing-reversed phase	Synergi Hydro-RP colum (2 mm x 100 mm, 2.1 µm); 10 mM tributylamine with 10 mM acetic acid (pH 5.5)/ methanol at a flow rate of 0,2 mL min^{-1} in the gradient elution mode	ESI(-)-QqQ-MS/MS, MRM mode; external standards calibration	[81]
2HG	Bone marrow aspirates from AML patients	Extraction with 80% (v/v) methanol /water at -80°C; centrifugation; supernatant evaporation to dryness; reconstitution in initial mobile phase	Ion pairing-reversed phase	Synergi Hydro-RP colum (2 mm x 100 mm, 2.1 µm); 10 mM tributylamine with 10 mM acetic acid (pH 5.5)/ methanol at a flow rate of 0,2 mL min^{-1} in the gradient elution mode	ESI(-)-QqQ-MS/MS, MRM mode; external standards calibration	[82]

Abbreviations: AML: Acute myeloid leukaemia; 2HG: 2-hydroxyglutarate; ESI(-)-QqQ-MS/MS: Electrospray ionization negative mode triple quadrupole tandem mass spectrometry; FFPE: formalin fixed paraffin embedded; IS: Internal standard; MRM: multiple reaction monitoring; PHEOs/PGLs: Pheochromocytomas/ paragangliomas; TCA: Tricarboxylic acid; SRM: Selected reaction monitoring.

Table 4.- Summarized performance characteristics among validated LC-MS methods for oncometabolite quantification in biological samples.

Sample	Oncometabolite	LOD (µM)	LOQ (µM)	[a]Precision (% RSD)		Recovery (%)	Reference
				Intra-day	Inter-day		
Human plasma	Succinate	0.06	0.18	8.1	18.9	No reported	[71]
	Fumarate	1.0	3.0	8.8	12.2		
Human serum, murine liver tissue and cultured cells	Succinate	0.099	0.198	2.51-19.51	2.20-19.92	100.68 serum 151.24 tissues 97.04 cells	[72]
	Fumarate	0.151	0.202	2.10-11.63	2.60-19.34	89.99 serum 77.24 tissues 102.54 cells	
Frozen and formalin-fixed paraffin-embedded kidney tissues	Succinate	No reported	0.02	6.2-14	No reported	[b]86-108	[73]
	Fumarate		0.2	3.4-14		[b]96-104	
	2HG		0.0020	3.9-13		[b]91-112	
Cultured cells	Succinate	0.0283	0.102	0.91-6.21	No reported	No reported	[75]
	Fumarate	0.0107	0.0388	0.56-6.80			

Abbreviations: 2HG: 2-hydroxyglutarate; LOD: limit of detection; LOQ: limit of quantification
[a]Precision expressed as percent of relative standard deviation (% RSD)
[b]Recovery assessed in pooled homogenates of FFPE or frozen tissues extracts

Table 5.- Summary of precolumn derivatization LC–MS methods for the determination of oncometabolites in biological samples.

Sample	Oncometabolite	Sample preparation	Derivatization reagent	Separation (column; mobile phase)	Detection ; quantification mode	LOQ (µM)	[a]Inter-day precision (% RSD)	Recovery (%)	Reference
Pig heart tissue	Fumarate	Tissue freeze-dry; extraction with ice-cold perchloric acid (0.6 M); centrifugation; supernatant adjust to pH 7.0; centrifugation; supernatant freeze-dry; reconstitution with 50% (v/v) acetonitrile/water; derivatization. On-line SPE clean-up using a C8 cartridge (2 mm × 4 mm, 5 µm)	MPEA	Kinetex Core–Shell C18 (2.1 x 100 mm ,2.6 µm); 0.1% formic acid in water/acetonitrile at a flow rate of 0.3 ml min^{-1} in gradient elution mode	ESI(+)-TOF-MS; external standards calibration	0.666	No reported	No reported	[84]
	Succinate					0.040	No reported	No reported	
Cultured cells and tumour xenografts	Fumarate	Addition of ISs (fumarate-^{13}C4, 2HG-d4, succinate-d4); extraction with ice-cold 80% (v/v) methanol/water; centrifugation; supernatant dried under a nitrogen stream; reconstitution with water; derivatization	O-BHA	Waters XBridge C18 column (2.1 x 50 mm) 0.1% formic acid in water/methanol at 0.6 ml min^{-1} in gradient elution mode	ESI(+)-MS/MS, MRM mode; isotopically labelled internal external calibration	0.0043	4.2- 11.3 (cells) 3.4- 8.4 (tumour)	90.9-97.5 (cells) 92.1- 106.2 (tumour)	[85]
	Succinate					0.0025	3.4- 5.2(cells) 2.4- 9.2 (tumour)	87.8- 90.1 (cells) 99.2- 105.6 (tumour)	
	2HG					0.0020	6.4- 11.4 (cells) 3.2- 10.5 (tumour)	90.9- 97.2 (cells) 94.3- 114.2 (tumour)	
Cultured cells, porcine skeletal muscle and heart tissues	Fumarate	Cells disruption and extraction with ceramic beads and 50% (v/v) methanol/water containing the ISs (fumaric acid-^{13}C4, succinic acid-d6, 2HG-d3); dried under speedvac; reconstitution; derivatization. Tissue freeze under dry ice; disruption and extraction with silicon carbide beads and 50% (v/v) methanol/water containing the ISs (fumaric acid-^{13}C4, succinic acid-d6, 2HG-d3); dried under speedvac; reconstitution; derivatization	4-BNMA	ChromXP-C18EP column (2.1 x 150 mm, 3 µm); 1% formic acid in water/acetonitrile at 0.025 ml min^{-1} in gradient elution mode	ESI(+)-MS/MS, MRM mode; isotopically labelled internal external calibration	0.0258	1	No reported	[86]
	Succinate					0.0085	0.5	No reported	
	2HG					0.614	5	No reported	
Dried blood spots	Fumarate	Addition of IS (citric acid-d4) and reagents for the derivatization; incubation for 1 h at 65 °C; stop reaction with mobile phase; centrifugation; supernatant analysis	DAABD-AE	Kinetex 1.7 µm C8 100A LC column (2.1mm x 50 mm); 0.05% perfluorooctanoic acid in water/methanol at 0.4 ml min^{-1} in gradient elution mode	ESI(+)-MS/MS, MRM mode; isotopically labelled internal external calibration	4.1	12.0- 18.4	No reported	[87]
	Succinate					2.0	12.3- 13.8	No reported	

Abbreviations: O-BHA: O-benzylhydroxylamine; 4-BNMA: 4-bromo-N-methylbenzylamine; DAABD-AE:4-[2-(N,N-Dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole; ESI(+)-TOF-MS : Electrospray ionization positive mode time of flight mass spectrometry; ESI(+)-MS/MS: Electrospray ionization positive mode time of flight tandem mass spectrometry; 2HG: 2-hydroxyglutarate; IS: Internal standard; LOQ: Limit of quantification; MPEA: N-methyl-2-phenylethanamine; MRM: Multiple reaction monitoring. [a]Inter-day precision expressed as percent of relative standard deviation (% RSD)

Table 6.- Summary of indirect LC-MS methodologies using chiral derivatization reagents for the determination of 2HG enantiomers in biological samples.

Sample	Sample preparation	Chiral derivatization reagent	Separation (column; mobile phase)	Detection ; quantification mode	LOQ (μM)	[a]Inter-day precision (% RSD)	Recovery (%)	Reference
Human urine	Addition of IS (D,L-2HG-^{2}H4); dilution with methanol; evaporation to dryness; derivatization	DATAN	Waters Xterra C18 (3.9 x 150 mm; 5 μm); 3.5 % (v/v) acetonitrile/water containing 125 mg·L^{-1} ammonium formate (pH adjusted to 3.6 with formic acid) at a flow rate of 0.75 mL min^{-1} in the isocratic elution mode	LC-ESI(-)-MS/MS, MRM mode; isotope-labelled internal standard calibration	1.0	6.2 L-2HG 5.9 D-2HG	91- 92 L-2HG 96-97 D-2HG	[95]
Human tissues	Frozen tissue homogenization in lysis buffer; addition of IS (D,L-2-hydroxyglutaric acid-d4); extraction twice with 80% (v/v) methanol/water precooled on dry ice; centrifugation; supernatant evaporation to dryness; derivatization	DATAN	Agilent Hypersil ODS (4 x 250 mm, 5 μm); 125 mg/l ammonium formate buffer (pH 3.6)/acetonitrile at a flow rate of 0.5 mL/min in the gradient elution mode	LC-ESI(-)-MS/MS, MRM mode; isotope-labelled internal standard calibration	No reported	No reported	No reported	[96]
Human serum	Extraction with methanol containing the	DATAN	Acquity UPLC BEH C18 (2.1 × 100 mm, 1.7 μm);	UPLC-ESI(-)-MS/MS, MRM mode; isotope-	No reported	No reported	No reported	[97]

	IS (D,L-2HG-d3); centrifugation; supernatant evaporation to dryness; derivatization		0.1% formic acid in 2mM ammonium formate/0.1% formic acid in methanol at a flow rate of 0.4 mL min^{-1} in the gradient elution mode	labelled internal standard calibration				
Cerebrospinal fluid	Filtration using 10 kDa membrane filters; filtrate collection; addition of IS (D,L-2-hydroxyglutaric acid-d4); evaporation to dryness; derivatization	DATAN	Waters XTerra MS C18 (3.9 x 150 mm; 5 µm); 7.0% (v/v) acetonitrile in ammonium formate buffer (pH 3.6)/acetonitrile at a flow rate of 0.4 mL min^{-1} in the gradient elution mode	LC-ESI(-)-MS, full scan mode; isotope-labelled internal standard calibration	0.1	4.4-17.9 L-2HG 3.7-13.8 D-2HG	93.2-109 L-2HG 95.6-107 D-2HG	[98]
Human serum	Addition of IS (D,L-2HG-d3); dilution with water; solid-phase extraction in an anion exchange and reversed phase STRATA^{TM}XL-A (200 mg–3 mL) cartridge; elution with methanol containing 0.1% formic acid; eluate evaporation to dryness; derivatization	DATAN	Agilent Zorbax SB®- C18 column (4.6 × 150 mm, 5µm); 2mM ammonium formate aqueous solution (pH 3.1 with formic acid)/ acetonitrile at a flow rate of 0.8 mL min^{-1}	LC-ESI(-)-MS/MS, MRM mode; isotope-labelled internal standard calibration	0.2	2.3-3.2 L-2HG 3.1-5.3 D-2HG	31.6-35.4 L-2HG 31.8-34.0 D-2HG	[99]
Human Serum	Addition of IS (D,L-2HG-d3); dilution with	DATAN	No reported	LC-ESI(-)-MS/MS, MRM	No reported	No reported	No reported	[100]

	water; solid-phase extraction in an anion exchange and reversed phase STRATA^{TM}XL-A (200 mg–3 mL) cartridge; elution with methanol containing 0.1% formic acid; eluate evaporation to dryness; derivatization			mode; isotope-labelled internal standard calibration				
Human urine and tissues	Urine: centrifugation; supernatant filtration with a Syringe Filter Nylon membrane (13 mm × 0.22 µ M); derivatization Tissue: homogenization and extraction with 80% (v/v) methanol/water pre-chilled at -80 ^{o}C; centrifugation; supernatant collection; derivatization	TSPC	Inertsil ODS-3 (2.0 x 250 mm, 5 µm); 0.1% formic acid in water/ 50% (v/v) methanol: acetonitrile at a flow rate of 0.2 mL min^{-1} in the gradient elution mode	LC-ESI(-)-MS/MS, MRM mode; standard additions calibration	0.002	2.8- 12.1 L-2HG 2.7-10.7 D-2HG	[b]93.9-110.9 L-2HG [b]88.0-109.5 D-2HG	[101]
Frozen and FFPE human tissues	FFPE tissue deparaffinization with xylene; deparaffinized FFPE or frozen tissue	TSPC	GL Sciences Inertsil-ODS3 column (2.1 × 250 mm, 5 µm); 0.1% formic acid in water/50% (v/v) methanol: acetonitrile at a flow rate of 0.2 mL min^{-}	LC-ESI(-)-MS/MS, MRM mode; isotope-labelled internal standard calibration	0.002	4.3-4.9 L-2HG 2.1-5.5 D-2HG	[c]89-96 L-2HG [c]94-96 D-2HG	[73]

	homogenization; addition of IS (D,L-2HG-d3); extraction twice with ice-cold methanol and 80% (v/v) methanol/water; centrifugation; supernatant collection; derivatization		[1] in the gradient elution mode					

Abbreviations: DATAN: diacetyl-L-tartaric anhydride; FFPE: Formalin-Fixed Paraffin-Embedded; 2-HG: 2-hydroxyglutaric acid; IS: internal standard; LC-ESI(-)-MS/MS: Liquid chromatography electrospray ionization negative mode tandem mass spectrometry; LOQ: limit of quantification; MRM: Multiple reaction monitoring; TSPC: N-(p-toluenesulfonyl)-L-phenylalanyl chloride.

[a]Inter-day precision expressed as percent of relative standard deviation (% RSD)

[b]Recovery obtained from urine

[c]Recovery obtained from pooled homogenate of FFPE or frozen tissues