

Metabolomic assays of the concentration and mass isotopomer distribution of gluconeogenic and citric acid cycle intermediates

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We developed gas chromatography-mass spectrometry assays for the relative concentration and for the mass isotopomer distribution of gluconeogenic and citric acid cycle intermediates in tissues. The assay involves (i) spiking the sample with one or more internal standards, (ii) chloroform-methanol extraction at -25°C , (iii) Folch wash of the extract, (iv) treatment of the water-methanol phase with methoxylamine, (v) evaporation and trimethylsilyl derivatization, and (vi) ammonia positive chemical ionization gas chromatography-mass spectrometry. For metabolomic computations, indices of concentrations for all compounds assayed are calculated as (Area of analyte)/(Area of reference compound). The assay was applied to a study of the effect of mercaptopicolinate, an inhibitor of phosphoenolpyruvate carboxykinase, on the profile of gluconeogenic intermediates in rat livers perfused with pyruvate. Crossover analysis of concentrations indices, compared to a control group, yielded very similar profiles as previous enzymatic assays, and correctly identified the site of action of mercaptopicolinate. Principal component analysis distinguished between control and drug treated samples. A loadings plot was used to identify the site of action of the drug in the metabolic pathway. Since metabolite concentrations do not address the flux through a pathway, perfusions with $[1,4-^{13}\text{C}_2]$ succinate dimethylester were conducted to assess fluxes around PEPCK. This allowed a dynamic metabolomics analysis which indicated that considerable flux through the pathway remained in the presence of mercaptopicolinate. This study illustrates the power of dynamic metabolomics to complement concentration based metabolomic studies.

KEY WORDS: mass spectrometry; gluconeogenesis; metabolomics; crossover analysis; citric acid cycle; stable isotopes.

Abbreviations: GC-MS: gas chromatography-mass spectrometry; TMS: trimethylsilyl; PCI: positive chemical ionization

1. Introduction

Classical metabolomics allows the identification of unexpected correlations between apparently unrelated pathways of metabolism (Fiehn, 2002). This strategy was elegantly demonstrated by Nicholson who showed that hydrazine intoxication of rats results in the inhibition of one step in lysine catabolism, resulting of the accumulation of a compound which blocks one step in tryptophan catabolism (Nicholls *et al.*, 2001). We hypothesized that the discovery potential of metabolomics could be greatly enhanced by associating it with mass isotopomer analysis of metabolites labeled from ^{13}C -labeled substrates (Katz *et al.*, 1993; Lee, 1993; Brunengraber *et al.*, 1997; Boros *et al.*, 2003). This association might allow to discover unexpected carbon fluxes between apparently unrelated pathways (Weckwerth and Fiehn, 2002). In view of the major developments in isotopomer analysis reported since the early

1990s, the association between metabolomics and mass isotopomer analysis is a natural step in the evolution of biological and biomedical investigation techniques.

In the course of our investigations on the interrelationship between gluconeogenesis and the citric acid cycle (CAC), we needed the ability to measure the concentration and ^{13}C -labeling of most intermediates of the two pathways. While such assays exist for CAC intermediates (Des Rosiers *et al.*, 1994), we are not aware of similar assays for most gluconeogenic intermediates. This is why we decided to set up such assays, geared for metabolomic investigations. Challenges to the setting up of mass spectrometric assays of phosphate esters are (i) their non-extractibility in organic solvents, (ii) their low concentrations in tissues, (iii) their relative instability, and (iv) their non-availability as ^{13}C -labeled standards. Also, in experiments with uniformly ^{13}C -labeled substrates, the intermediates of both the CAC and gluconeogenesis become labeled with multiply-labeled mass isotopomers. This would preclude the use of $[\text{U}-^{13}\text{C}]$ standards if they were available. Wu *et al.*

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have reported the biological preparation of multiply-labeled internal standards by incubating yeast with [$U\text{-}^{13}\text{C}_6$]glucose and [$U\text{-}^{13}\text{C}_2$]ethanol (Wu *et al.*, 2005). This mixture of almost uniformly labeled internal standards would be suitable for assaying the concentration of metabolites from experiments with unlabeled or moderately labeled substrates.

In the present study, we explored the possibility of assaying the profile of concentration and mass isotopomer pattern of water-soluble metabolites, using a mixture of three reference compounds, i.e., [$U\text{-}^{13}\text{C}_6$]citrate, [$U\text{-}^{13}\text{C}_4$]succinate, and 3-hydroxy- $[\text{}^2\text{H}_5]$ glutarate. The metabolites, extracted by chloroform-methanol followed by Folch wash (Folch *et al.*, 1951), were derivatized with methoxylamine and trimethylsilyl before GC-MS analysis. The analytical procedures were tested in rat livers perfused with pyruvate \pm mercaptopicolinate (MPA), an inhibitor of phosphoenolpyruvate (PEP) carboxykinase (Jomain-Baum *et al.*, 1976).

2. Methods

2.1. Materials

Chemicals, biochemicals, and enzymes were obtained from Sigma-Aldrich, except for mercaptopicolinic acid which was obtained from Apin Chemical Ltd (UK). [$U\text{-}^{13}\text{C}_6$]Citrate, [$U\text{-}^{13}\text{C}_4$]succinate (99%), $^2\text{H}_2\text{O}$ and NaO^2H (99%) were purchased from Isotec (Miami, OH). [$1,4\text{-}^{13}\text{C}_2$]succinic acid (99%) and NaB^2H_4 (99%) were purchased from Cambridge Isotope Laboratories, Inc. R,S -3-hydroxy- $[\text{}^2\text{H}_5]$ glutarate was prepared by isotopic exchange of α -ketoglutarate in $^2\text{H}_2\text{O}$ and NaO^2H , followed by reduction with NaB^2H_4 , and extraction in acid. This procedure is similar to that used previously for the preparation of R,S -3-hydroxy- $[\text{}^2\text{H}_5]$ butyrate from acetoacetate (Des Rosiers *et al.*, 1988). The purity of R,S -3-hydroxy- $[\text{}^2\text{H}_5]$ glutarate was assessed by GC-MS and NMR. [$1,4\text{-}^{13}\text{C}_2$]Succinate dimethylester was prepared by reacting the acid with excess diazomethane. The purified product was free of monoester and free acid.

2.2. Liver perfusion experiments

Sprague-Dawley male rats were kept for 8–12 days on Teklad F6 rodent chow *ad libitum*. Livers from overnight-fasted rats (180–220 g) were perfused (Brunengraber *et al.*, 1975) (40 ml/min) with non-recirculating Krebs-Ringer bicarbonate buffer containing 2 mM pyruvate \pm 0.3 mM mercaptopicolinate. Since the mercaptopicolinate molecule contains a free SH group, we wanted to avoid the possibility of oxidation in the perfusate gassed with 95% O_2 + 5% CO_2 . Therefore, mercaptopicolinate was infused into the liver perfusion line

via a syringe pump. After 40 min, the livers were quick-frozen, and kept in liquid N_2 until analysis. Two other livers were perfused with 0.3 mM mercaptopicolinate and 0.5 mM of either [$1,4\text{-}^{13}\text{C}_2$]succinate or [$1,4\text{-}^{13}\text{C}_2$]succinate dimethylester.

2.3. Sample preparation (figure 1)

Powdered frozen tissue (1.5 g), spiked with reference compounds (150 nmol of [$U\text{-}^{13}\text{C}_6$]citrate, 100 nmol of [$U\text{-}^{13}\text{C}_4$]succinate, and 50 nmol of R,S -3-hydroxy- $[\text{}^2\text{H}_5]$ glutarate) is extracted with 19 mL of chloroform-methanol 2:1, pre-cooled at -25°C , using a Polytron homogenizer. During the 5 min extraction, the tube is partially immersed in acetone kept at -25°C by periodical addition of dry ice. Then, 6 mL of ice-cold water is added to the tube, and the extraction is continued for 5 min (Folch, *et al.*, 1951). The slurry is centrifuged at 670 g for 20 min at 4°C . The upper methanol-water phase is collected and treated with 200 μmol of methoxylamine-HCl to protect keto and aldehyde groups. The lower chloroform phase is vortexed for 5 min with 10 mL of methanol-water 3:2 pre-cooled at -20°C . After 20 min centrifugation at 4°C , the two upper methanol-water phases are combined. If needed, the chloroform phase can be used for lipid analyses. The combined methanol-water phase is adjusted to pH 8.0 with NaOH, and is evaporated in a Savant vacuum centrifuge. The residue is reacted with 100 μL of bis(trimethylsilyl) trifluoroacetamide with 10% trimethylchlorosilane (Regisil) at 70°C for 50 min, to form the TMS and MOX-TMS derivatives of the analytes.

2.4. GC-MS conditions

Analyses were carried out on an Agilent 5973 mass spectrometer, equipped with a model 6890 gas chromatograph, autosampler, a Varian VF-5MS capillary column (60 m, 0.25 mm i.d., 0.25 mm film thickness), and a EZ guard column (10 m). The carrier gas was helium with a pulse pressure of 40 psi. Helium flow through the column was constant at 1 ml/min. The injection was either 1 μL split 10:1, or 2 μL splitless. The injector temperature was set at 270°C and the transfer line at 280°C . The 70 and 30 min GC temperature programs were: (i) start at 80°C , hold for 1 min, increase by $3^\circ\text{C}/\text{min}$ to 320°C , hold at 320°C for 5 min; (ii) start at 80°C , hold for 1 min, increase by $10^\circ\text{C}/\text{min}$ to 320°C , hold at 320°C for 5 min. The ion source was set at 150°C and the quadrupole at 150°C . The ammonia pressure was adjusted to optimize peak areas. For each analyte, we monitored the signals at the nominal m/z (M_0) and at all detectable naturally labeled mass isotopomers (M_1 , M_2 , M_3). The mass isotopomer distribution of each analyte was compared to the theoretical distribution calculated with a computer program.

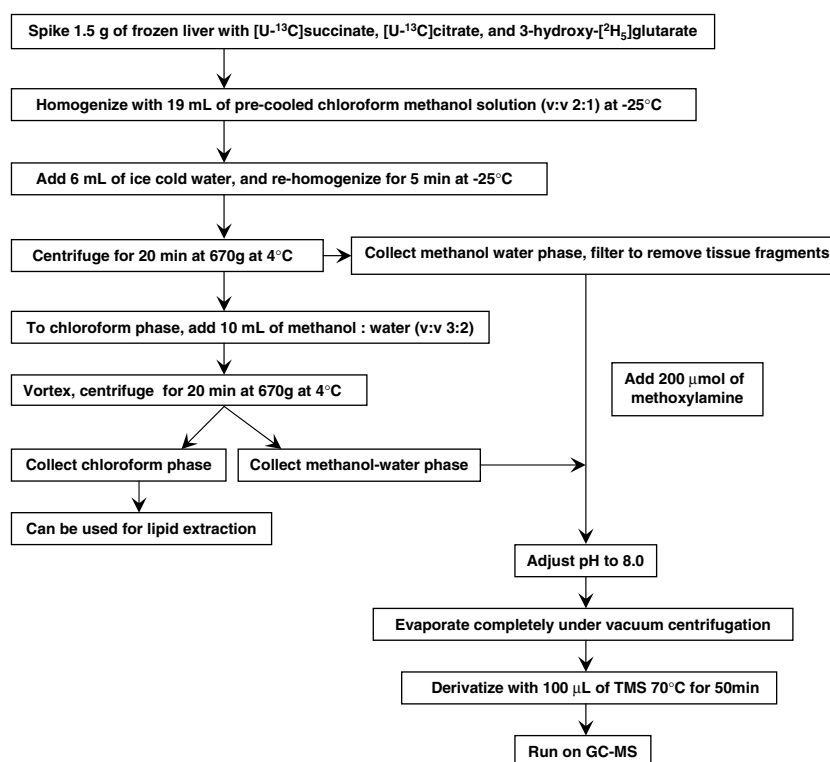


Figure 1. Procedure for tissue extraction.

The assays were also conducted under electron ionization conditions. The fragmentation pattern of analytes was introduced in the NIST (National Institute of Science and Technology) software to help with the identification and to detect interferences. Unlabeled standards of all analytes were assayed to confirm the identity of compounds assayed in tissue extracts.

To check for the linearity of relative concentrations, we assayed increasing amounts of one batch of frozen liver powder (0.4 to 2 g), spiked with the same amounts of the three reference compounds.

2.5. Calculations

Raw mass spectrometric areas were used to calculate the analytical parameter (area of analyte)/(area of reference compound). This parameter is not a relative concentration. However, it can be used to calculate ratios of analyte concentrations in comparisons between control and intervention groups, using crossover analysis (Chance, 1959). This is because the area of the reference compound cancels itself out in the following equation:

Relative concentration

$$= \frac{\text{Average} [(\text{Area of analyte})/(\text{Area of reference compound})]_i}{\text{Average} [(\text{Area of analyte})/(\text{Area of reference compound})]_c}$$

where i and c represent the intervention group and the control group, respectively. The formula was used to

calculate three sets of relative concentrations using each of the three reference compounds.

Principal Component Analysis (PCA) was performed on mean centered metabolite data using Mathcad, (Mathsoft, Cambridge, MA). To complete the array for missing data, a normally distributed random number with standard deviation of the relevant metabolite was supplied to the PCA routine. The data analysis also included a loadings plot showing the correlations between the original variable and the principal component, reflecting the relative contributions/importance of each variable in each principal component.

3. Results

In orientation experiments, we tested the analyses with a short and a long temperature gradient in the gas chromatograph (30 and 70 min). We found that derivatives of some of the phosphoesters of gluconeogenesis (dihydroxyacetone phosphate, glyceraldehyde-3-phosphate, and 2-phosphoglycerate) were identifiable with the short, but not the long temperature gradient. Presumably, the derivatives of these phosphoesters are temperature-labile and are not stable during prolonged heating. The other metabolites of gluconeogenesis and the CAC were identifiable with either gradient. Table 1 shows the retention times of the metabolites under both gradients. Because of our interest in gluconeogenic phosphoesters, we selected the short gradient for all analyses reported below.

Table 1
Retention times (RT) and m/z monitored for the assays of citric acid cycle and gluconeogenic intermediates

	RT (min) of short program	RT (min) of long program	PCI ions monitored
Pyruvate-MOX-TMS	4.43	12.9	207–210
Glycerol- TMS ₃	7.90	21.9	309–312
Succinate- TMS ₂	8.55	23.8	280–284
Fumarate- TMS ₂	9.04	25.6	278–282
OAA-MOX- TMS ₂	10.46	29.9	323–327
Malate- TMS ₃	10.72	30.9	368–372
Aspartate-TMS ₃	11.13	32.5	350–354
α KG-MOX- TMS ₂	11.86	34.6	337–342
PEP- TMS ₃	12.11	35.4	385–388
DHAP-MOX- TMS ₃	13.72	ND	416–419
GA3P-MOX- TMS ₃	13.84	ND	416–419
α GP- TMS ₄	13.85	41.3	461–464
2PG- TMS ₄	14.06	ND	475–478
3PG- TMS ₄	14.36	42.9	475–478
Citrate- TMS ₄	14.48	43.3	481–487
Glucose- TMS ₅	16.03	48.5	558–564
F6P-MOX- TMS ₆	18.56	56.9	603–609
G6P-MOX- TMS ₆	19.67	60.5	603–609

Note: MOX: methoxylamine; OAA: oxaloacetate; α KG: alpha-ketoglutarate; DHAP: dihydroxyacetone phosphate; GA3P: glyceraldehyde-3-phosphate; α GP: alpha glycerol phosphate; 2PG: 2-phosphoglycerate; 3PG: 3-phosphoglycerate; F6P: fructose-6-phosphate; G6P: glucose-6-phosphate. ND: not detectable.

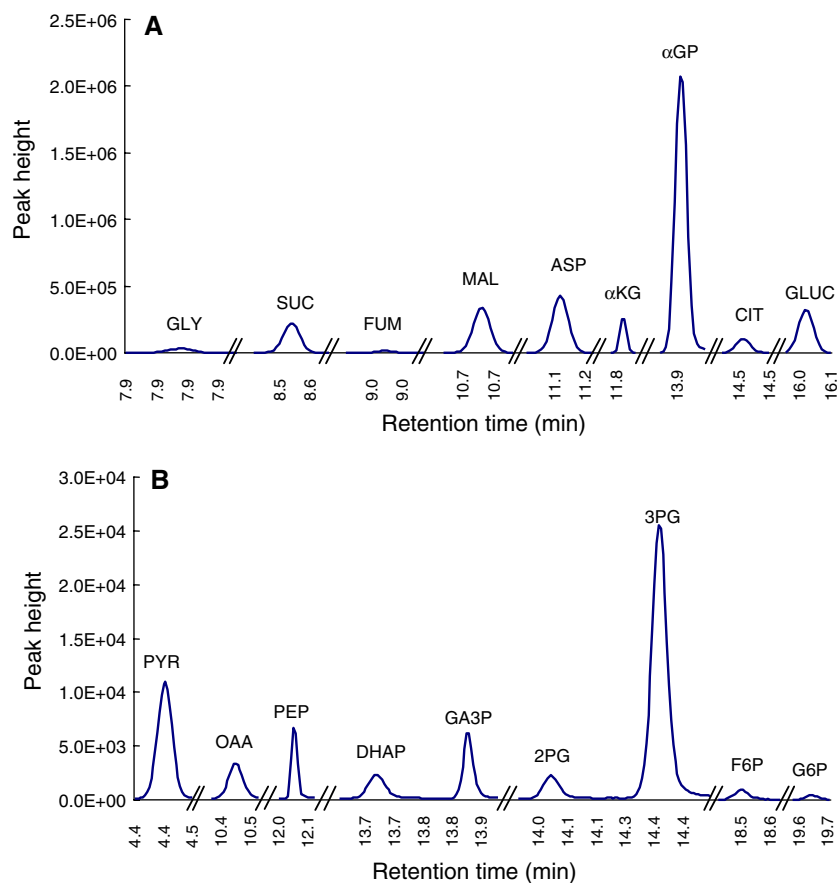


Figure 2. Positive chemical ionization chromatograms of metabolites extracted from one liver perfused with 5 mM lactate. Extraction and derivatization were conducted as outlined in figure 1. The two chromatograms were obtained by injecting different volumes of the same derivatized extract (Panel A: 1 μ L, split 10:1; Panel B: 2 μ L, splitless).

Figure 2 panels A and B show the chromatograms of metabolites extracted from a perfused rat liver, under split and splitless conditions, respectively. The retention times and m/z monitored were first determined using pure standards. Under ammonia positive chemical ionization and selective ion monitoring (table 1), the peaks showed distributions of naturally labeled mass isotopomer identical to theoretical distributions. In addition, under electron ionization, the identity of the analytes was confirmed by the NIST software on the basis of fragmentation patterns. Interference was identified: at M2 of PEP and M2 of fumarate (with both gradients, under ammonia positive chemical ionization, but not under electron ionization). This interference needs to be dealt with in experiments with ^{13}C -labeled substrates leading to M2 labeling of PEP and fumarate.

Figure 3 shows the crossover analysis (Chance, 1959) of compounds assayed in rat livers perfused in the absence and presence of 0.3 mM mercaptopicolinate, an inhibitor of PEP carboxykinase (Jomain-Baum *et al.*, 1976). The data, for each analyte, are presented as relative concentrations, compared to controls (see Methods). A crossover is evident at PEP carboxykinase, as had been previously shown using enzymatic assays (Blackshear *et al.*, 1975).

To test for the linearity of the assay of the analytical parameter (area of analyte)/(area of reference compound), we analyzed increasing amounts of one batch of frozen liver powder (0.4 to 2 g), spiked with the same amounts of the three reference compounds. For each analyte, we plotted the ratios as a function of the amount of tissue analyzed, and calculated the coefficient of correlation R^2 (table 2). R^2 values ranged from 0.81 to 0.998.

The study reported here quantified 12 targeted metabolites in a well known area of mammalian metabolism in response to a drug whose site of inhibition in the pathway has been thoroughly documented.

Looking forward, an important goal of metabolomic investigations will be to find the site action of novel drugs. With this in mind, we examined the present data as a prototype for a novel drug and asked whether unsupervised PCA could distinguish between the two classes of samples (control and drug treated). PCA indicates that over 96% of the variation in the data can be captured by the first two principal components. The PCA scores plot (figure 4A) further indicates that the two treatment groups are well separated. The loadings plot (figure 4B) illustrates the individual metabolites that contributed most to PC1 and PC2. On this plot the positive and negative vectors indicate metabolites that are increased and decreased by the drug, respectively, and suggest a site of inhibition in the pathway between the CAC and the triose phosphates.

Profiles of steady state levels of metabolites as obtained may be considered “static metabolic” data because the metabolite levels do not provide information about fluxes in the pathway. In contrast “dynamic” metabolomic data may be obtained by including isotopically labeled compounds in the analysis. We investigated the mass isotopomer distribution of CAC and gluconeogenic intermediates in two livers perfused with 0.3 mM mercaptopicolinate and 0.5 mM of either $[1,4-^{13}\text{C}_2]$ succinate or $[1,4-^{13}\text{C}_2]$ succinate dimethylester (figure 5). This was an orientation experiment for another study where we investigated the homogeneity of labeling of intermediates in perfused rat livers. The goal of this orientation experiment was to compare the labeling of intermediates from $[1,4-^{13}\text{C}_2]$ succinate and $[1,4-^{13}\text{C}_2]$ succinate dimethylester. Rognstad had reported that rates of gluconeogenesis in rat hepatocytes are greater with succinate dimethyl ester than with succinate salt, because of the poor permeability of the latter through the cell membrane (Rognstad, 1984). The data clearly show that $[1,4-^{13}\text{C}_2]$ succinate dimethylester is more effective at labeling intermediates

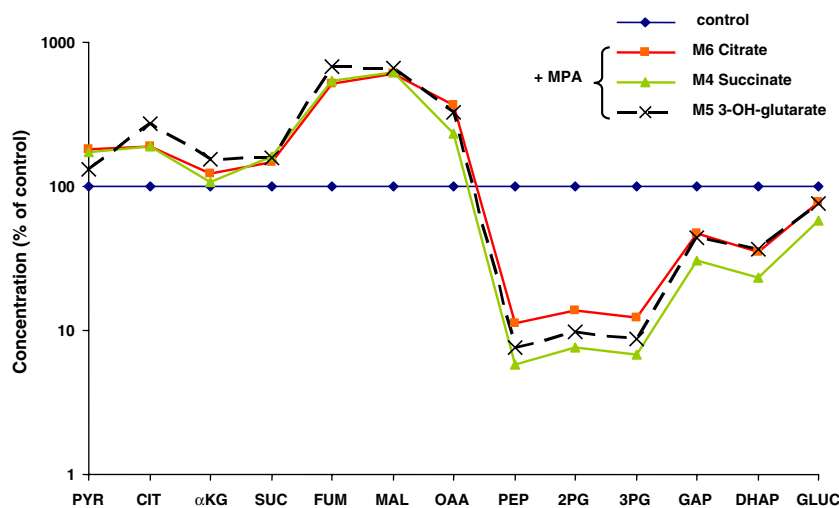


Figure 3. Crossover analysis of CAC and gluconeogenic intermediates extracted from livers perfused with 2 mM pyruvate \pm 0.3 mM mercaptopicolinate.

Table 2

Comparison of coefficients of correlation (R^2) of the linear regressions of the assays of citric acid cycle and gluconeogenic intermediates with 3 reference compounds

R^2	PYR	CIT	α KG	SUC	FUM	MAL	OAA	2PG	3PG	PEP	GA3P	DHAP	GLUC
[U- 13 C $_4$] Succinate	0.99	0.97	0.96	0.998	0.94	0.96	0.92	0.84	0.98	0.98	0.88	0.81	0.93
[U- 13 C $_6$] Citrate	0.98	0.98	0.94	0.99	0.95	0.97	0.87	0.85	0.99	0.99	0.91	0.84	0.97
3-Hydroxy-[2 H $_5$]glutarate	0.98	0.94	0.97	0.94	0.93	0.99	0.89	0.89	0.99	0.99	0.85	0.86	0.98

Note: PYR: pyruvate; CIT: citrate; α KG: alpha-ketoglutarate; SUC: succinate; FUM: fumarate; MAL: malate; OAA: oxaloacetate; 2PG: 2-phosphoglycerate; 3PG: 3-phosphoglycerate; PEP: phosphoenolpyruvate; GA3P: glyceraldehyde-3-phosphate; DHAP: dihydroxyacetone phosphate; GLUC: glucose.

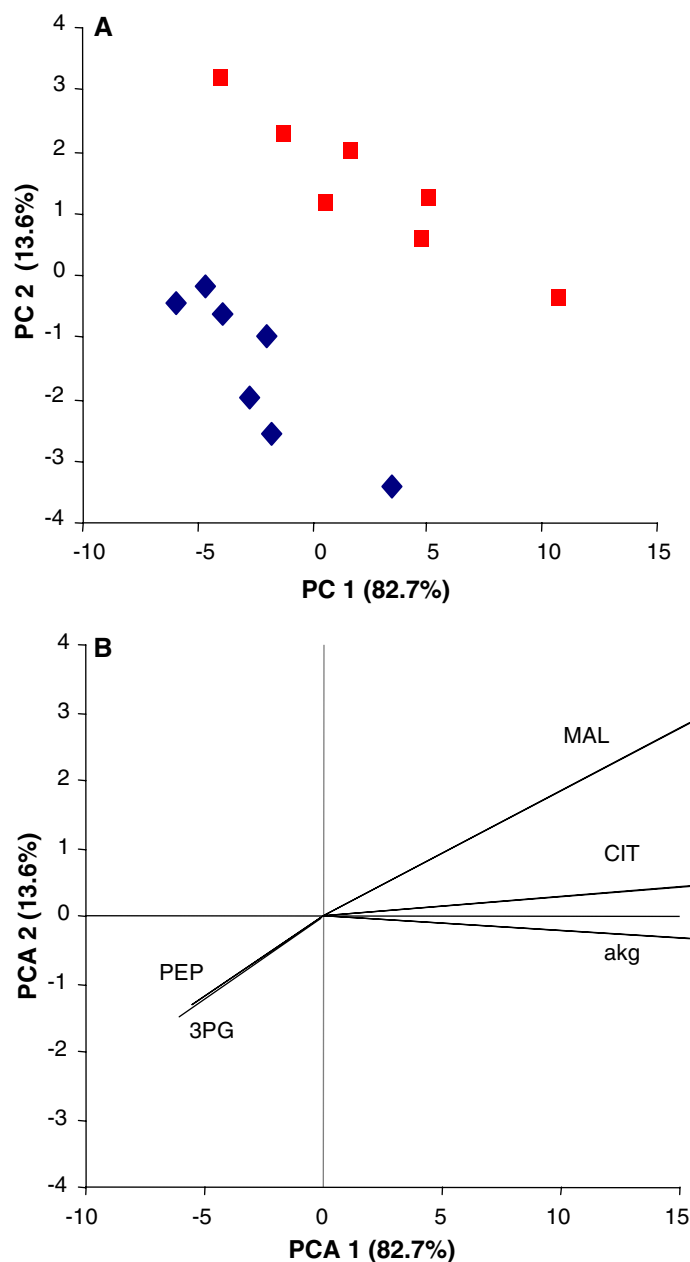


Figure 4. Principal component analysis of the data of the liver perfusions with 2 mM pyruvate \pm MPA (same data as used for figure 3). Panel A: PCA scores plot for individual samples indicating percent of variance explained by each PC. The plot represents the 7 control samples (diamonds) and the 7 MPA-treated livers (squares). Panel B: Graphical representations of the contributions (loadings) of the original variables to PC 1 and PC 2. These are normalized on the graph to an arbitrary scale reflecting the relative values of the component score coefficients.

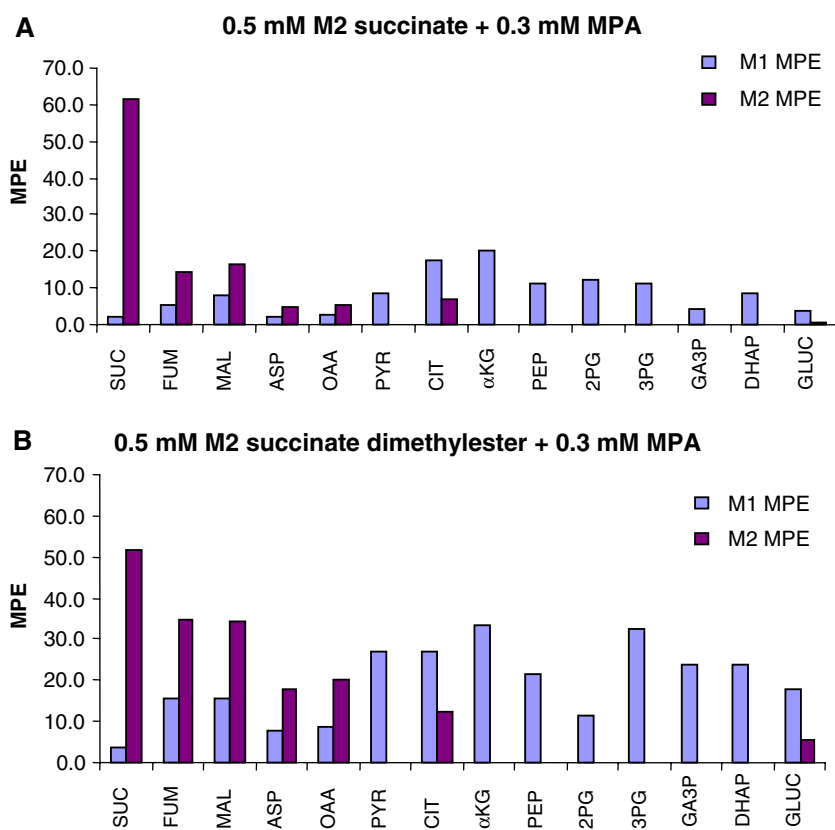


Figure 5. Comparison of mass isotopomer distributions of metabolites extracted from two livers perfused with 0.3 mM mercaptopicolinate and either 0.5 mM $[1,4-^{13}\text{C}_2]$ succinate or 0.5 mM $[1,4-^{13}\text{C}_2]$ succinate dimethylester.

than $[1,4-^{13}\text{C}_2]$ succinate. This confirms that the uptake of the non-ionized succinate diester is greater than that of succinate salt as found by Rognstad (Rognstad, 1984). In addition, the labeling data provides insight into the flux patterns that produce the metabolites. Examination of the $[1,4-^{13}\text{C}_2]$ succinate dimethylester

data indicates that labeled C entering the CAC as $[1,4-^{13}\text{C}_2]$ succinate provides an estimate of the pyruvate cycling consisting of the sum of two fluxes (oxaloacetate \rightarrow PEP \rightarrow oxaloacetate and malate \rightarrow pyruvate \rightarrow malate) relative to the CAC (figure 6). We previously used the term “*R*” to denote the probability

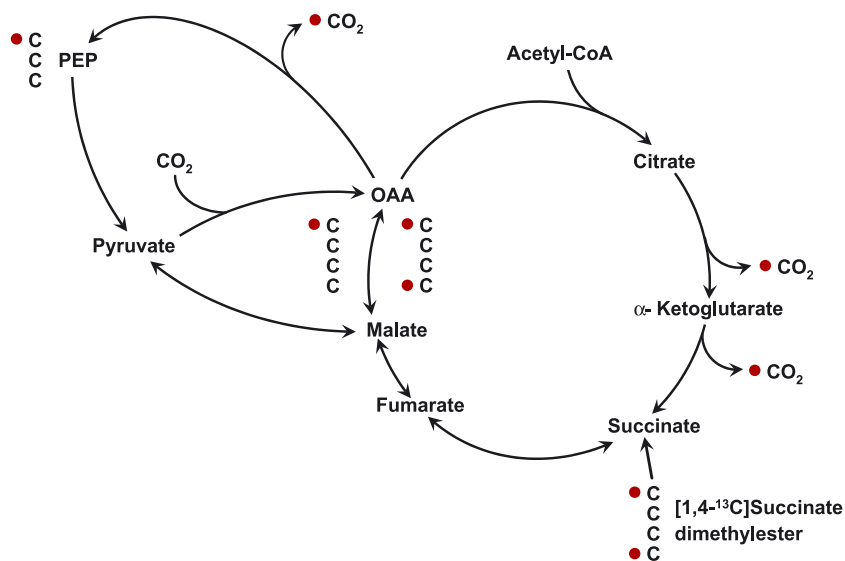


Figure 6. Fate of $[1,4-^{13}\text{C}_2]$ succinate dimethylester in the CAC. The figure emphasizes the cycling of labeled intermediates between oxaloacetate, PEP, pyruvate, and malate.

that a molecule of oxaloacetate will travel this cycle rather than move forward in the CAC. Using principles developed previously (Kelleher, 1985) we derived the following equation for R :

$$R = \frac{2 M1}{2 M2 + M1}$$

where the terms M1 and M2 refer to the mass isotopomer distribution of malate, oxaloacetate and compounds in equilibrium with them. As indicated in this equation, the presence of M1 malate or oxaloacetate indicates flux through pyruvate cycling. Using the data from figure 5B, the value for R was nearly constant (0.36–0.37) for each of the four carbon intermediates, oxaloacetate, fumarate malate and aspartate. Thus, these four compounds are near isotopic equilibrium in the CAC. Additionally, this data indicate that, despite the presence of 0.3 mM mercaptopycolinate, substantial pyruvate cycling flux remains, equivalent to 36–37% of the CAC rate. Some M1 four-carbon intermediates may result from the action of malic enzyme but the finding of labeled glucose can only result from PEP carboxykinase flux and gluconeogenesis (figure 5B). Thus, stable isotopes add information that cannot be obtained through static metabolomics, as had been also hypothesized out by Boros *et al.* (2003).

4. Discussion

The present study is part of the development of metabolomic techniques for the assay of the concentration and mass isotopomer distribution of large numbers of metabolites in biological fluids and tissues. We chose the chloroform–methanol extraction followed by Folch wash (Folch *et al.*, 1951) for the following reasons. First, enzymes are rapidly inactivated by chloroform. Second, conducting the first extraction of frozen tissue powder at -25°C prevents metabolic changes before enzymes are inactivated. Third, the extraction procedure does not add salts to the preparation. Fourth, the Folch wash yields a (i) water–methanol phase containing, presumably, most water-soluble metabolites, and (ii) a chloroform phase containing, presumably, most lipids. This is an advantage for studies of water-soluble metabolites. Fifth, the water–methanol phase is pH neutral and can be treated immediately, if needed, with methoxylamine or another protector of keto and aldehyde groups.

The identification of a metabolite in a metabolomic GC-MS profile involves usually two criteria, (i) identity of retention time with that of a pure standard injected alone in a parallel run, and (ii) high probability of identity of the fragmentation pattern of the analyte with that of data banks such as that of the National Institute of Standards and Technology (NIST). In the present

study, we have used a third criterion, i.e., the identity of the mass isotopomer distribution of the analyte with that of a pure standard and the theoretical distribution, when the data are collected under ammonia chemical ionization conditions. Because of the natural occurrence of (i) ^{13}C and ^{15}N atoms in many metabolites and derivatization agents, and (ii) heavy isotopes of silicon in silylating derivatization agents, the mass spectrum of molecules and fragments is made of clusters of masses associated with the nominal mass of the ion. The natural mass isotopomer distribution of the molecular ion, assayed under chemical ionization, is also a “fingerprint” of the compound. This is why the identification of a compound in our GC-MS runs requires the identity of the mass isotopomer distribution of the analyte assayed in the biological matrix and in a single standard run. Extending the criteria of identification to the whole mass isotopomer distribution allows detecting contaminant(s) which would artifactually increase or decrease the M1, M2...enrichments of the analyte in experiments with ^{13}C - or ^2H -labeled substrates. An artifactual decrease or increase in enrichment would occur if the contaminant yielded a signal at the nominal mass (M) or at one or more of the higher satellite masses (M1, M2, M3), respectively.¹

In metabolomic investigations, it is difficult and, in many cases impossible to (i) use labeled internal standards for all compounds to be analyzed, and (ii) to run calibration curves for the concentration of all analytes. Wu *et al.* proposed to use, as a mix of internal standards, an extract of yeast cells grown on $[\text{U-}^{13}\text{C}_6]\text{glucose} + [\text{U-}^{13}\text{C}_2]\text{ethanol}$ (Wu, *et al.*, 2005). Thus, the labeled metabolome was used as internal standards for the unlabeled metabolome. Presumably, under these growth conditions, most yeast metabolites are uniformly labeled, with a small proportion of mass isotopomers the mass of which is one *amu* less than the fully labeled isotopomer. This is because commercial labeled substrates are prepared from 99% ^{13}C and 98% ^2H -building blocks. One could conceivably use an extract of labeled yeast cells as an internal standard mixture for assaying the concentration of metabolites in mammalian cells, organs, or body fluids. However, this strategy would not be applicable to the measurement of both the concentration and the mass isotopomer distribution of metabolites in experiments where metabolites are heavily labeled with stable isotopes.

In metabolomic investigations involving one control group and one or more intervention groups, the effect of the intervention can be assessed without measuring absolute concentrations of metabolites. One does not even need to calculate true relative concentrations such as (concentration of analyte)/(concentration of an unnatural

¹ Mass isotopomers are designated as M_i , where i is the number of atomic mass units above the molecular weight of the unlabeled isotopomer M.

standard added to the preparation before extraction). One can study the effect of the intervention with the parameter (area of analyte)/(area of one reference compound). There are two types of suitable reference compounds which can be used for the assay of many metabolites: (i) an unnatural compound such as 3-hydroxy- $^{2}\text{H}_5$ glutarate, or tricarballic acid, or (ii) a uniformly labeled natural compound, such as $[\text{U-}^{13}\text{C}_6]$ citrate (M6). The latter is usable in experiments without stable isotopes, or in experiments with stable isotopes where only M1 to M3 mass isotopomers of citrate are formed.

The parameter (area of analyte)/(area of one reference compound) can be used for Principal Component Analysis (Dillon and Goldstein, 1984), or for crossover analysis (Chance, 1959) as we did in the present study. Then, one calculates for each analyte (i) the average parameter (area of analyte)/(area of reference compound) for each group, and (ii) the ratio (average parameter of intervention group)/(average parameter of control group). The latter ratios are plotted as in figure 3 which shows the effect of mercaptopicolinate on the reactions of gluconeogenesis from pyruvate. The similarity of the crossover analysis plots generated from the three reference standards demonstrates that, for the type of study presented, one can use a single reference compound to identify the effect of an intervention on a series of biochemical reactions. This is spite of the differences on chemical functional groups of the compounds investigated (phosphoesters, carboxylates, hydroxycarboxylates, ketocarboxylates). The crossover plots clearly identifies the reaction catalyzed by PEP carboxykinase as the flux-generating step of gluconeogenesis from lactate. The plot is almost identical to figure 6 of (Blackshear *et al.*, 1975) where samples from a similar experiment were analyzed by enzymatic assays.

The above findings (figure 3) do not imply that the setting up of metabolomic assays using a single reference compound can be conducted without calibration curves. It is important to demonstrate that the analytical parameter (area of analyte)/(area of reference compound) varies linearly with the amount of analyte being assayed. This is why, in a separate experiment, we assayed increasing amounts of liver powder (0.4–2 g) with the same amount of the three reference compounds used in the analyses of figure 3. Table 2 shows that the R^2 of the linear regression assays ranged from 0.81 to 0.99. This range is similar to the range reported by Wu *et al.* (0.86 to 0.99) for calibration curves based on unlabeled standards (Wu *et al.*, 2005).

Relative concentrations (intervention group) *vs.* (control group) can be assayed even when the metabolites are extensively labeled with ^{13}C , since the reference compound is not affected by the labeling of analytes. However, the analytical parameter must be calculated using the sum of the areas of all the detected mass isotopomers of the analyte:

$$\text{Parameter} = (\sum \text{Area}_i \text{ of analyte}) / (\text{Area reference compound})$$

where i refers to each detected mass isotopomer, including the unlabeled nominal mass M . For the reference compound, one can just use the area of the nominal mass.

The ^{13}C labeling data (figure 4) provide additional insight into the actions of 0.3 mM mercaptopicolinate, not apparent from the static metabolomic data. As emphasized above, the static metabolomic indicated inhibition of the pathway at the conversion of oxaloacetate to PEP. However, the crossover plot provides no information about flux. In contrast, the isotopic data indicates that substantial flux through PEP carboxykinase remains after treatment with the inhibitor. The increase in metabolite levels for the upstream precursors PEP, observed in the crossover plot, does not necessarily represent a block in the flux at that point. Instead, it may represent an increase in the flux control coefficient for PEP carboxykinase through the actions of the irreversible inhibitor, MPA. As a result of the inhibitor binding a fraction of the enzyme molecules, the substrate, oxaloacetate increases and flux through the pathway continues. The isotopic data clearly show that some PEP carboxykinase flux continues in the presence of mercaptopicolinate. Thus an increased flux control coefficient for PEP carboxykinase is likely to be one effect of the inhibitor in this preparation. This finding is consistent with previous work by Groen and co-workers who estimated that the PEP carboxykinase flux control coefficient in liver was low, approximately 0.08, in the absence of inhibitor (Groen *et al.*, 1982). The use of inhibitors to alter metabolite concentrations and affect metabolic control has been discussed in more detail by Fell (Fell, 1997). Here we demonstrate the potential of dynamic metabolomics to complement more typical static metabolomic profiles.

Figure 5 illustrates the application of our assay technique to a study of the mass isotopomer distribution of liver metabolites. This orientation experiment was conducted to compare the efficiency of labeling of CAC and gluconeogenic metabolites from identical concentrations of either $[1,4\text{-}^{13}\text{C}_2]$ succinate or $[1,4\text{-}^{13}\text{C}_2]$ succinate dimethylester. With the exception of succinate, the labeling of all assayed metabolites was greater from $[1,4\text{-}^{13}\text{C}_2]$ succinate dimethylester than from $[1,4\text{-}^{13}\text{C}_2]$ succinate (figure 5). This results from the greater permeability of the non-ionized diester compared to the divalent salt. Our data are compatible with those of Rognstad who reported a higher rate of gluconeogenesis from succinate dimethyl ester compared to succinate salt (Rognstad, 1984). The lower labeling of succinate in figure 5A *vs.* 5B can be explained as follows. The di-TMS derivative assayed corresponds to free succinate in the liver extract. About one-half of the mass of liver

extracted corresponds to the extracellular fluid which contained [1,4-¹³C₂]succinate in the first liver, and mostly [1,4-¹³C₂]succinate dimethyl ester in the second liver. In the effluent of the latter liver, we detected 99% M2 labeled [1,4-¹³C₂] succinate monomethyl-TMS esters (not shown). Thus, in the second liver, the free succinate peak assayed resulted from the hydrolysis of the dimethyl ester. In both livers, the enrichment of 99% labeled [1,4-¹³C₂]succinate entering the CAC was diluted by succinate of lower labeling recycled in the CAC. This dilution was decreased in the extract of the first liver by the presence of [1,4-¹³C₂]succinate in the extracellular fluid. A detailed study of the metabolism of [1,4-¹³C₂]succinate in perfused rat livers will be presented in a subsequent paper.

In conclusion, we present an analytical technique which allows to study variations in the concentration and mass isotopomer distributions of gluconeogenic and CAC intermediates in two GC-MS runs of the same derivative mix, with different split ratios. Although the concentration data are not expressed in absolute units, the analytical parameter (area of analyte)/(area of reference compound) can be used to identify variations resulting of an intervention and, in cases such as shown in figure 3, to identify the site of action of inhibitors in a metabolic pathway. PCA conducted on mean centered data for control and drug-treated livers clearly distinguished between the two classes. Furthermore, scores plots indicated the site of action of the drug. This study combining static and dynamic metabolomics may provide a guide for developing metabolomic protocols for finding the targets of novel drugs.

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