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An optimized protocol for metabolome analysis in yeast using direct infusion electrospray mass spectrometry

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Abstract

A method for the global analysis of yeast intracellular metabolites, based on electrospray mass spectrometry (ES-MS), has been developed. This has involved the optimization of methods for quenching metabolism in *Saccharomyces cerevisiae* and extracting the metabolites for analysis by positive-ion electrospray mass spectrometry. The influence of cultivation conditions, sampling, quenching and extraction conditions, concentration step, and storage have all been studied and adapted to allow direct infusion of samples into the mass spectrometer and the acquisition of metabolic profiles with simultaneous detection of more than 25 intracellular metabolites. The method, which can be applied to other micro-organisms and biological systems, may be used for comparative analysis and screening of metabolite profiles of yeast strains and mutants under controlled conditions in order to elucidate gene function via metabolomics. Examples of the application of this analytical strategy to specific yeast strains and single-ORF yeast deletion mutants generated through the EUROFAN programme are presented.

Keywords: Yeast; Quenching and extraction method; Intracellular metabolite analysis; Electrospray mass spectrometry (ES-MS); Direct infusion; Metabolic profiles; Metabolic fingerprinting; Metabolome; Metabolomics; Functional genomics

1. Introduction

Strategies for the determination of intracellular metabolites, either for specific compounds or different families of intermediary metabolites have been reported in the past for different organisms (e.g. yeast, bacteria, and plants; Bhattacharya et al., 1995; Buchholz et al., 2001; De Koning and van Dam, 1992; Fiehn et al., 2000b; Gancedo and Gancedo, 1973; Saez and Lagunas, 1976). With the emergence of new fields of research such as metabolic engineering and metabolic control analysis (MCA; Edwards et al., 2001; Fell, 1997; Fiehn, 2001; Weckwerth and Fiehn, 2002), many new methods and techniques are being developed that allow the study and comparison of sets of metabolic profiles acquired by different analytical approaches (e.g. target analysis, metabolite profiling and metabolic fingerprinting; Fiehn, 2002; Fiehn et al., 2000a; Glassbrook et al., 2000; Vaidyanathan et al., 2001). Functional genomics is a field of study that is initially directed at elucidating the function of unknown genes (Delneri et al., 2001; Kell and Mendes, 2000; Oliver, 1996; Oliver et al., 1998; Ter Kuile and Westerhoff, 2001; Tretheway et al., 1999) and has generated a need to develop new strategies for the analysis of the complete pool of cellular metabolites (the 'metabolome'; Fiehn, 2002; Oliver, 1997; Oliver et al., 2002; Phelps et al., 2002; Raamsdonk et al., 2001; Teusink et al., 1998; Tweeddale et al., 1998).

The different factors involved and the requirements for appropriate analysis of intracelullar metabolites (e.g fast sampling, quenching, and efficient extraction of metabolites) have been reported by a number of authors (De Koning and van Dam, 1992; Lange et al., 2001; Saez and Lagunas, 1976). Once the metabolites have been extracted, different analytical techniques can be applied for their qualitative and quantitative determination. The following methods have sufficient sensitivity and are the most commonly used, either alone or combined with

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different chromatographic methods: enzymatic and immunological methods, NMR, and mass spectrometric techniques (Fell, 1997). However, the major limitation of metabolic profiling for functional genomics is the need for sensitive, high-throughput methodologies for global analysis and screening of a large number of samples (Oliver et al., 2002). The current state of the technology means that different metabolite profiling techniques have to be combined in order to identify and quantify all of the metabolites in a biological system (Tolstikov and Fiehn, 2002).

In this study, a simple method for quenching metabolism and extracting metabolites (Gonzalez et al., 1997) has been optimized for the direct and reproducible analysis of the yeast metabolome by electrospray mass spectrometry (ES-MS; Gaskell, 1997; Vaidyanathan et al., 2001). Attention is focused on: the use of defined cultivation conditions in a synthetic defined medium; the influence of sample size; fast sampling; quenching and extraction at neutral pH, using non-salt-based (NSB) buffers; and the influence of storage, for efficient ES-MS analysis of metabolite samples.

2. Results and discussion

2.1. Optimization of cultivation conditions

Initial studies using complex media (YPD) yielded poor reproducibility, presumably because the composition and properties of complex media may vary depending on the batch or the manufacturer, and also on the storage and autoclaving conditions (Chatterjee et al., 2000). In order to improve reproducibility and detection of slight differences between mutants, a defined medium (Baganz et al., 1997) was selected. The preparation and autoclaving of the medium were controlled to ensure maximum reproducibility (see Experimental). Exponential-phase cultures ($A_{600}=1.0$) were selected to study the influence of the different variables on the quenching and extraction methods.

2.2. Optimization of quenching and extraction steps: the choice of buffer

The efficacy of extraction of different groups of metabolites may vary according to both the pH and the particular buffer system employed. The majority of metabolic studies with yeast recommend the use of a neutral pH for quenching and extraction (Gonzalez et al., 1997; Saez and Lagunas, 1976). However, although there are a number of buffers with pK_a in the range 6.5–8.0, the reality is that the majority of these buffers are composed of salts (e.g. phosphate, HEPES, PIPES). This may cause undesirable ion suppression effects in mass spectrometry studies (Gaskell, 1997; Sterner et al., 2000). In some other cases, the buffers may be toxic, or they may undergo degradation at 80 °C, which precludes their use for extracting metabolites at high temperatures (see Experimental section). In preliminary studies, HEPES and imidazole buffers were discarded for these reasons, and tricine ($M_r=179.2$) was finally adopted as an adequate non-salt-based (NSB) buffer for metabolite profiling and optimization of the method. Examples of ES-MS metabolic profiles using salt-based buffers (HEPES) with presence of ion suppression effects, and tricine under different conditions are shown in Fig. 1.

2.3. Influence of evaporation step and sample volume

The concentration of intracellular metabolites may vary depending on different cell states and conditions (Gancedo and Gancedo, 1973; Saez and Lagunas, 1976). Thus, under aerobic conditions, yeast metabolites such as glycolytic intermediates, amino acids, and nucleotides are normally present at mM concentrations. Other metabolites (e.g. vitamins, or glycolytic intermediates under anaerobic conditions) may approach the μM range (De Koning and van Dam, 1992; Martinez-Force and Benitez, 1991; Raamsdonk, 2000). The extraction of metabolites from the intracellular volume to a final volume of extraction solvent necessarily entails their dilution. Therefore, the size of the sample (in mg, dry weight, of biomass) taken and the concentration step that raises the level of metabolites in the extract above the detection limit of the instrument used in their final analysis have to be optimized. In the majority of cases, this concentration step is performed by the evaporation of the extraction solvent under vacuum (De Koning and van Dam, 1992; Gonzalez et al., 1997; see Experimental).

The influence of both the evaporation step and sample size is illustrated in Fig. 1. Low levels of biomass and the absence of a concentration step results in a low number of peaks, with low intensities (Fig. 1A). However, when more cells (30 mg dry wt) were used, and an evaporation step included (see Experimental), a significant number of intracellular metabolites could be detected (Fig. 1B), with more than 25 intrinsic metabolites being found in the range 100–1500 m/z. From these results, the main groups of metabolites are expected to correspond to the different groups of extracted metabolites reported by Gonzalez et al. (1997). Thus, peaks in the low m/z range (below 400) can be attributed to glycolytic intermediates and specific amino acids, whereas some of the peaks in the high m/z range (above 600) are more likely to correspond to nucleotides, pyridine nucleotides (NADH, NADPH), and cofactors (CoA, acetyl-CoA). For a precise elucidation of all present metabolites more detailed studies leading to the identification of specific peaks have to be performed. This is presently under investigation.

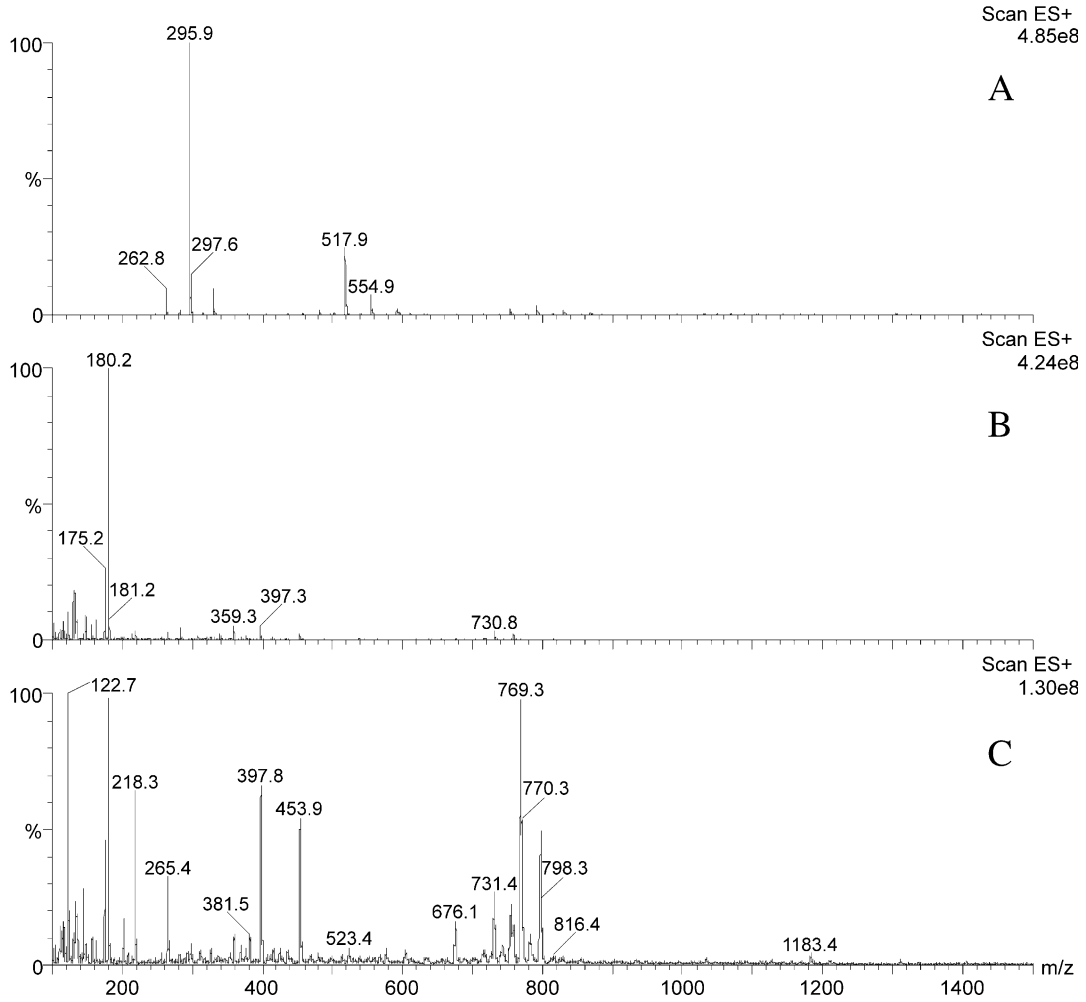

Fig. 1. Electrospray mass spectra of low molecular weight intracellular metabolites of *S. cerevisiae* Y03925 growing in exponential phase (A_{600} = 1.0). A) 10 mg dry wt, using HEPES (0.25 mM, pH = 7.5 in ethanol, 75% (v/v), without concentration step; B) 10 mg dry wt, using tricine (0.5 mM, pH = 7.4 in ethanol, 75% (v/v), without concentration step; C) 30 mg dry wt, with tricine, including concentration step.

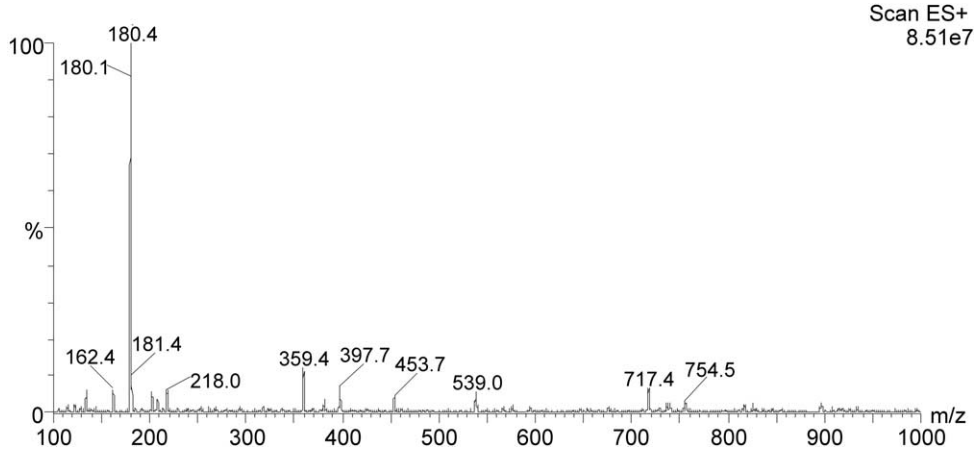

Fig. 2. Electrospray mass spectrum of extraction solution (used as a blank, no cells present) following same treatment (extraction, concentration and preparation of samples for ES-MS analysis) (see Experimental).

The initial sample size, the evaporation step, and the volume of sample injected for ES-MS analysis were optimized to enable detection of metabolites in the mM physiological range (see Experimental), with final concentrations in the extracts above the μM detection limit of the ES-MS (see Experimental and Section 2.4, mass spectrometry studies).

2.4. Mass spectrometry (ES-MS) studies

Some previous studies have reported that electrospray ionisation techniques have limited utility for the quantitative analysis of metabolites due to undesirable effects such as ion suppression (Sterner et al., 2000; Weckwerth and Fiehn, 2002). In our study, we intended to use direct infusion of unfractionated cell extracts into the mass spectrometer, initially for first qualitative analyses. Our optimization of the culture medium, sample volume, quenching, extraction, and concentration steps have enabled us to produce spectra with a large number of peaks that provide a useful picture of intracellular metabolites that is not compromised by any detectable ion suppression effects (Weckwerth and Fiehn, 2002). The use of a defined medium, a washing step, and the absence of polar buffers are all major factors in the minimisation of ion suppression effects. Our procedures may be used for the high-throughput comparison of metabolic profiles and fingerprinting (Fiehn, 2002; Vaidyanathan et al., 2001).

2.5. Detection limits and the leakage of metabolites from cell samples

An experiment was performed to determine whether metabolites leaked from the cells in the first quenching step. For that purpose, after quenching 30 mg of washed cells, the volume of quenching solution was evaporated and analysed by ES-MS. This resulted in peaks of negligible intensities compared to the real metabolic profiles (results not shown), in accordance with previous studies that reported a limited extraction capacity of quenching solutions at low temperatures (De Koning and van Dam, 1992).

We decided to evaluate the number of detectable peaks coming from either the extraction solvent or the

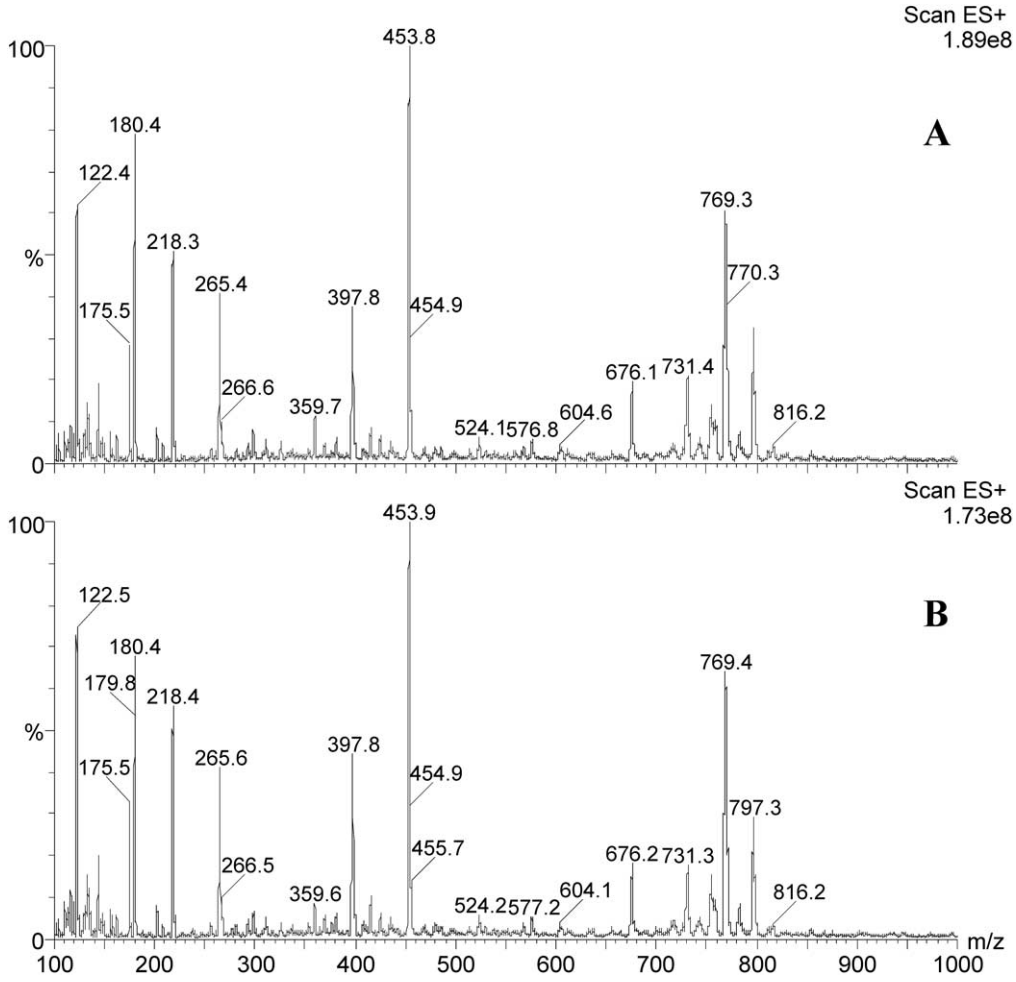

Fig. 3. Reproducibility studies. Electrospray mass spectra from independent experiments of quenching and extraction of metabolites of *S. cerevisiae* Y03925 growing in exponential phase ($A_{600} = 1.0$). Starting cell material: 30 mg.

walls of the tubes (50 ml Falcon tubes) in which the cells were harvested. To accomplish this an extraction step (80 °C, 3 min) was carried out in the absence of yeast cells and ES-MS analysis was performed on the extraction solvent alone. The results (Fig. 2) show a small number of peaks which, although of low intensity, would routinely be included in our metabolic profiles (see Experimental section).

2.6. Reproducibility, storage, and stability

Experiments were performed in triplicate and produced highly reproducible results. Fig. 3 shows the metabolic profiles of two independent experiments in which we quenched metabolism in *S. cerevisiae* strain Y03925 and extracted metabolites for analysis by ES-MS. The two spectra are essentially identical, in terms of the number, m/z values, and relative heights of the peaks. Thus, the procedure is qualitatively reproducible and should also enable the design of quantitative experiments in the future.

Finally, a study of the influence of storage on the stability of samples was performed. Profiles of samples, following evaporation of the extraction solvent and storage at −80 °C for at least 4 weeks, were very similar to those obtained from the original samples. This agrees with earlier conclusions that negligible alterations in metabolite levels occur during storage at −80 °C (Fiehn, 2002). However, when ES-MS samples that had been prepared in acetonitrile/water plus formic acid (see Experimental) were stored at −20 °C, significant changes in the profiles showing degradation of specific peaks were observed after 1 week. The protocol for the preparation of ES-MS samples was adapted in the light of these results (see Experimental).

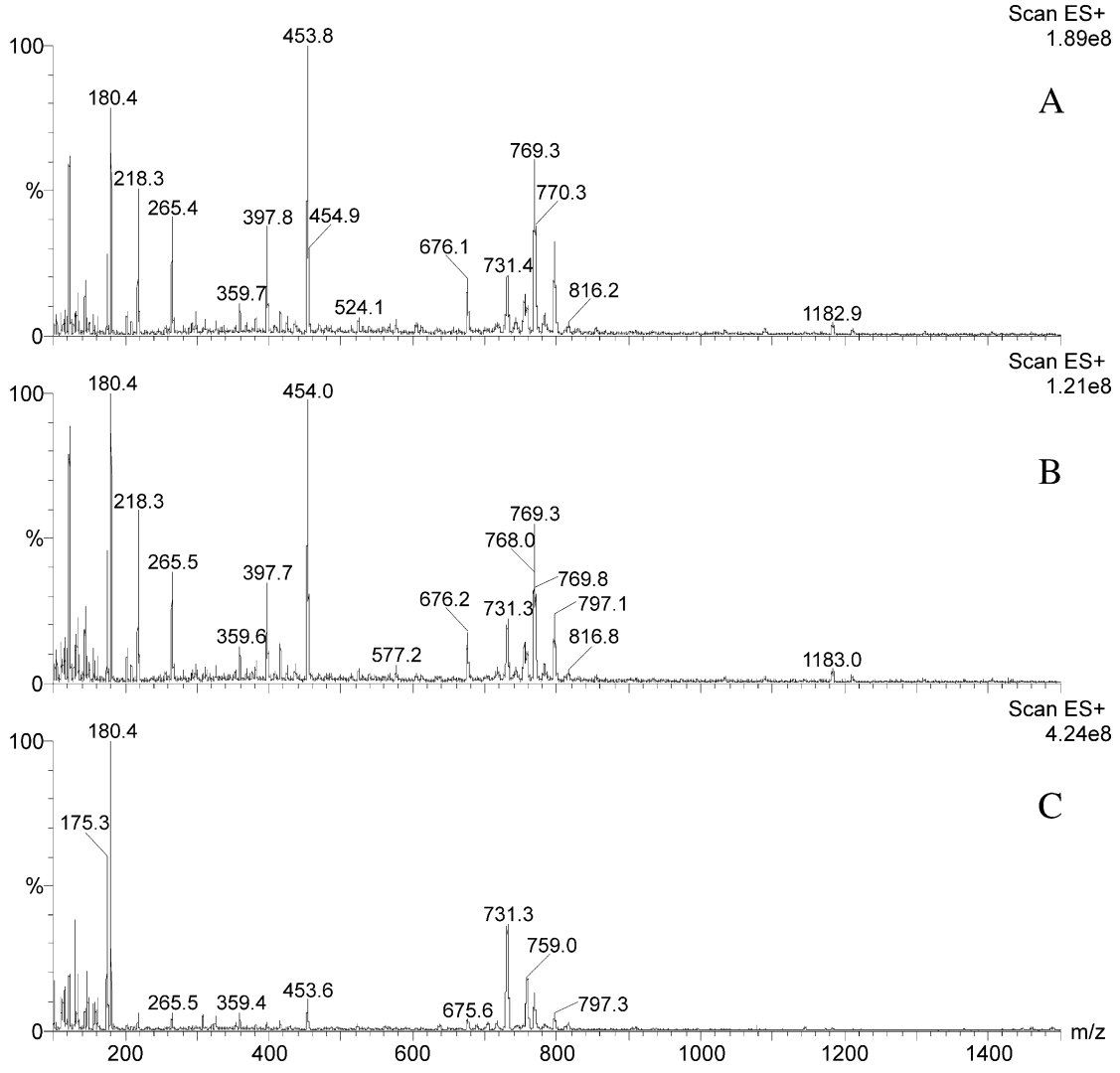

Fig. 4. Electrospray mass spectra of intracellular metabolites (whole m/z range, 100–1500) of: (A) *S. cerevisiae* Y03925 (reference strain); (B) Y05397 (mutant disrupted in a non-essential gene of unknown function; ORF YNR022c); (C) *S. cerevisiae* BY4709. All cultures were grown to mid-exponential phase ($A_{600} = 1.0$). The conditions of analysis were as in Fig. 3.

2.7. Studies with yeast mutants

The methodological strategy for quenching and analysis of metabolites can be applied to the study of metabolic profiles of different mutant strains in systematic functional genomics studies. We have compared the metabolic profiles of three *S. cerevisiae* strains that are all direct derivatives of the S288C strain that is commonly used by the yeast genetics community, and whose genome was sequenced (Goffeau et al., 1996). The three strains studied are all members of the BY lineage (Brachmann et al., 1998). They include BY4709, which contains a single genetic marker (*ura3Δ*) and two deletion mutants created in the multiply marked BY4741 derivative that was used for the yeast genome deletion project (Giaever et al., 2002; Winzeler et al., 1999). One of these (Y03925) contains the *ho::kanMX4*, which is known to be without phenotypic effect at either the level of growth rate (Baganz et al., 1998) or the metabolome (Oliver et al., 1998; Raamsdonk et al., 2001), while the other (Y05397) is deleted for ORF YNR022c, which defines a non-essential gene of unknown function. The results (Figs. 4 A–C) show clear differences between the metabolic profiles of the singly (Fig. 4C) and multiply marked (Figs. 4A and B) strains. This emphasises the critical importance of marker effects in physiological studies on yeast (Pronk, 2002). A comparison of the spectra for Y03925 and Y05397 reveal very similar metabolic profiles, with only slight (but reproducible) differences in the relative contribution of very small peaks. It is thus essential that comparisons be made between completely isogenic strains that both contain the replacement marker (kanMX). We have previously established that *ho::kanMX* strains represent a useful standard for such comparisons (Baganz et al., 1998; Oliver et al., 1998; Raamsdonk et al., 2001).

A method of extraction directed to metabolic profiling and Functional Genomics should maximize the proportion of metabolites, representing all functional categories, which can be extracted simultaneously. However, this ideal goal cannot be achieved with a unique method since the complete pool of intracellular metabolites (the 'metabolome') comprises a wide spectrum of compounds with different chemical properties (e.g. low-molecular-weight compounds, sugars, lipids, amino acids and peptides, nucleotides, vitamins and cofactors).

In this study a first global method for extraction of a representative number of metabolites (between 25 and 40) has been optimized for direct analysis by electrospray mass spectrometry (ES-MS). The method is capable of detecting a broad spectrum of metabolites of different functional categories (glycolytic intermediates, nucleotides, pyridine nucleotides, amino acids and organic compounds. (Gonzalez et al., 1997). Thus, our optimized method can be applied for global analyses, involving the screening of a large number of strains., Very small differences can be detected and interpreted with the aid of specific data analysis and bioinformatic methods (e.g. supervised learning methods, and genetic programming; Gilbert et al., 1997; Kell and King, 2000; Raamsdonk et al., 2001). For exhaustive metabolomics studies, the method should be augmented with other techniques and strategies (e.g. liquid chromatography coupled to ES-MS), which,will allow highly sensitive, comprehensive analysis, identification and quantification of metabolites.

3. Conclusions

We have previously demonstrated the feasibility of using metabolomics analysis to uncover the functions of genes with no overt phenotype (Raamsdonk et al., 2001). We have now sought to develop protocols that are sufficiently fast, facile, robust, and efficient that they would enable the analysis of the thousands of mutants generated in the yeast genome deletion project (Giaever et al., 2002; Winzeler et al., 1999). A method of quenching and extraction of metabolites has been optimized to allow efficient extraction of intracellular metabolites from *Saccharomyces cerevisiae* and their analysis by ES-MS. The ES-MS technique has several advantages over the NMR analysis that we employed previously (Raamsdonk et al., 2001), including an analysis time of just 2 min per sample and automated procedures for peak assignment. Characteristic metabolic profiles with more than 25 specific internal metabolites can be detected by ES-MS. Metabolic profiles show good reproducibility allowing the comparison of the metabolomes of yeast mutants and 'reference strains' that are applicable in functional genomics studies (e.g. multivariate FANCY; Raamsdonk et al., 2001) under controlled conditions. Future studies will include application of the method to sets of mutants of known and unknown genes related to specific functional categories under different culture conditions. Specific data mining methods (i.e. supervised methods and genetic programming methods) will enable the elucidation of the physiological function of unknown genes (Kell et al., 2001).

Finally, in functional genomics, the importance of rigorous experimental design (such that as many confounding variables as possible are removed from the system) is of paramount importance. Careful selection of the specific strains to be compared and their culture under physiologically well-defined and rigorously controlled conditions (e.g. chemostat culture) may result in significant biological insights being made from metabolomics analyses. These, in turn, may be related to other levels of functional genomic analysis (e.g. transcriptome

and proteome) in an integrative approach (Delneri et al., 2001; Oliver et al, 2002). Bioinformatic tools that permit the integration of qualitatively and quantitatively disparate sets of functional genomic data have been developed (Paton et al., 2000) and exploited in validation and integration of genetic, transcriptomic, and proteomic data (von Mering et al., 2002). The integration of metabolomics data on the complete set of 6000 yeast single-ORF deletion mutants, garnered using the robust and facile protocols described in this paper, will greatly enhance comprehensive analyses of gene action and interaction in this important model organism and contribute to a more holistic view of eukaryotic cell biology.

4. Experimental

4.1. Reagents

The defined medium was prepared using analytical grade reagents. Solutions for quenching, extraction and ES-MS analysis were prepared using water and organic solvents of HPLC grade. All other reagents and chemicals were of the highest purity available.

4.2. Yeast strains

Saccharomyces cerevisiae strain ATCC 200872 and yeast strains from the genome deletion project (EUROSCARF collection, Frankfurt. http://www.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html) were selected for optimization of the method and study of metabolic profiles. The strains are presented in Table 1.

4.3. Medium and cultivation conditions

Experiments were performed in triplicate in batch cultures (250 ml Erlenmeyer flasks containing 50 ml of culture volume) in a defined synthetic medium (F1; Baganz et al., 1997) with a glucose concentration of 20 g/l and an initial pH of 5.0. Glucose was autoclaved separately from the other medium components. Vitamins and nutritional requirements (histidine, leucine, uracil and methionine, final concentration 0.1 g/l) were prepared in a concentrated form, sterilized by filtration (0.2 μm pore size cellulose acetate filters; Millipore) and added to the medium after autoclaving. Exponential-phase cultures ($A_{600} = 1.0$; equivalent to 0.8 mg/ml of biomass) growing at 30 °C were selected for quenching and extraction of metabolites and analysis of metabolic profiles.

Table 1
S. cerevisiae haploid strains from the genome deletion project (SGD, EUROSCARF)[a,b] and the ATCC

Strain (accession No.)	Genotype
BY4709 (ATCC 200872)	*MAT*α *ura3Δ*
BY4741 (Y03925)	*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ho::kanMX4*
BY4741 (Y05397)	*MAT*a *his3Δ leu2Δ0 met15Δ0 ura3Δ0* YNR022c*::kanMX4*

[a] http://www.unifrankfurt.de/fb15/mikro/euroscarf/col_index.html.
[b] http://mips.gsf.de/proj/yeast/CYGD/db/index.html.

4.4. Quenching and extraction of intracellular metabolites

The method of quenching and extraction of metabolites of Gonzalez et al. (1997) was optimized in terms of sampling, quenching and extraction conditions, to enable the efficient extraction of metabolites and their direct analysis by electrospray mass spectrometry (ES-MS). Firstly, for the preparation of quenching and extraction solutions different buffer systems were tested. Best results were obtained with tricine, a non-salt-based (NSB) buffer, and this was selected for further studies. Quenching and extraction solutions were prepared fresh (less than 48 h old) from sterile concentrated solutions of tricine at pH = 7.4 , and stored at 4 °C prior to use. (a) Quenching solution: methanol 60% (v/v); 10 mM buffer; pH = 7.4, used in quenching and washing steps. (b) Extraction solution: ethanol 75% (v/v); 0.5 mM buffer; pH = 7.4.

4.4.1. Sampling, quenching and washing

Direct quenching of the culture in the appropriate volume of solution (Gonzalez et al., 1997) at −50 °C was performed by fast sampling (De Koning and van Dam, 1992; Gonzalez et al., 1997; Lange et al., 2001; Theobald et al., 1993) of a volume of culture equivalent to (a) 10 mg or, (b) 30 mg dry weight, the latter being adopted as the standard sample size for the optimized protocol. Thus, assuming maximal extraction efficiency, 30 mg of biomass, cytosolic volume of 3.75 μl per mg of protein, 1 mg of yeast protein being equivalent to 2.5 mg dry weight (De Koning and van Dam, 1992; Raamsdonk et al., 2001) the whole method results in an overall dilution factor of ca. 17. This allows for groups of metabolites in the mM physiological range (e.g. 0.1–5 mM) (De Koning and van Dam, 1992; Gancedo and Gancedo, 1973; Saez and Lagunas, 1976) to fall within the range of detection by ES-MS (i.e. μM range).

Fast sampling was performed using an automatic pipette (Pipetboy acu, Integra Biosciences, Letchworth, UK). The quenching step was performed at −50 °C for 3 min, in 50 ml Falcon tubes (Fisher Scientific, UK). Temperature was kept at −50 °C ± 2 °C by controlled addition of dry ice to a laboratory dry ice/ethanol bath monitored continuously with a digital thermometer. After the quenching step, in order to remove residual traces of external medium, cells were centrifuged, washed and vortexed with the same quenching solution

at −50 °C (10 ml quenching solution per 50 ml Falcon tube). Centrifugation was performed at 7500 rpm, for 3 min with the rotor pre-cooled to −20 °C, and the centrifuge controlled to the same temperature. The temperature of the solution was checked to certify that it remained well below −20 °C after the centrifugation.

4.4.2. Extraction and evaporation steps

Extraction was performed in 50 ml Falcon tubes containing 5 ml of extraction solvent per tube, in a water bath at 80 °C for 3 min (Gonzalez et al., 1997). Samples were then concentrated and dried by evaporation using a Speedvac concentrator vacuum system, SPD111V, connected to a Micromodulyo Freeze Dryer (Thermo-Life Sciences, Basingstoke UK). After evaporation of the solvent, samples were stored at −80 °C, prior to ES-MS analysis. Metabolite samples were stable at −80 °C for a number of weeks. However, where possible, samples were analysed immediately following the evaporation step.

4.5. Electrospray mass spectrometry (ES-MS) analysis

Preparation of samples for mass spectrometry was performed immediately prior to ES-MS analysis, and after calibration of the mass spectrometer as described below.

For the preparation of the samples, metabolites from evaporated samples were resuspended in 400 μl of ethanol (75% v/v). For the ES-MS analysis, 50 μl of each sample were mixed with 50 μl of acetonitrile/water (1:1, v/v, 0.1% HCOOH). Positive-ion mode electrospray mass spectra were obtained using a Quattro II mass spectrometer (Micromass, Manchester, UK), source conditions were optimized for analytes. Samples were introduced at a flow rate of 5 μl/min. The mass analyser was scanned at a rate of 100 Th s^{-1} and the data acquisition and processing were controlled by the Masslynx data system (Micromass, Manchester, UK). Acquired mass spectra represented sums of 10–30 scans. Mass calibration was performed using a calibration mixture composed of *N*-acetyl-lysine, myoglobin, and bradykinin fragment 2-9 (2 μM each).

4.6. Experimental design and data processing

Metabolic profiles of mutants of genes of unknown function can be compared to the metabolic profiles of mutants of genes of known function (reference strains) from the same genetic background. Essential genes should be studied using heterozygous diploid mutants. For a first study with non-essential genes, haploid strains were selected (Table 1). Metabolic profiles were analysed using MassLynx 3.3 and the results compared against the corresponding reference strain and control (see section 2).

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