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Metabolite profiling of sprouting mung beans (*Vigna radiata*)

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Abbreviations

CI	Confidence interval
CV	Column volume
DW	Dry weight
EI	Electron impact ionization
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization of the United Nations
FID	Flame ionization detection
g	Gram
GC	Gas chromatography
GM	Genetically modified
h	Hour
ha	Hectare
HPLC	High performance liquid chromatography
I.D.	Inner diameter
InsP	Inositol phosphate
min	Minute
ml	Milliliter
mm	Millimeter
MS	Mass spectrometry
MSTFA	N-methyl-N-trimethylsilyltrifluoroacteamide
MTBE	Methyl-*tert*-butylether
mu	Mass units
NMR	Nuclear magnetic resonance
OECD	Organization for Economic Co-operation and Development
PC	Principal component
PCA	Principal component analysis
RI	Refraction index
RT	Retention time
s	Second
SD	Standard deviation
SPE	Solid phase extraction

TIC	Total ion count
TMS	Trimethylsilyl-
TMSIM	Trimethylsilylimidazole
UPLC	Ultra performance liquid chromatography
WHO	World Health Organization

1 INTRODUCTION AND OBJECTIVES

The plant kingdom is estimated to contain 200 000 compounds (Fiehn, 2002; Dixon and Strack, 2003) with up to 20 000 metabolites present in a single plant (Bino et al., 2004; Fernie et al., 2004). Metabolomics, an unbiased profiling approach, has been shown to be a powerful analytical platform for the investigation of plant and crop metabolomes. The metabolic phenotype in a biological system is mainly defined by the genetic background (e.g. different species), breeding strategies (e.g. genetic engineering), environmental conditions (e.g. growing location, season), induced stress (e.g. drought, salinity, pathogens), farming practices (e.g. low input system) and the developmental stage. During the past decade, numerous metabolomics-based investigations have been conducted in plants in order to assist in functional genomics, to analyze metabolic pathway interactions of primary and secondary plant constituents and to elucidate nutrition and health-related quality traits which enabled the breeding-driven metabolic engineering of important nutrients. Capillary gas chromatography-based metabolite profiling approaches in combination with powerful statistical tools have been proven to provide suitable platforms for comprehensive investigations of plant-derived foods. As examples, GC-based metabolite profiling (i) assisted in the elucidation of mutation events in rice (Frank et al., 2007) and soybean (Frank et al., 2009), (ii) allowed comparative analyses of Bt- and Roundup Ready-maize with the respective isogenic lines in the light of environmental influences (Frank et al., 2012a) and (iii) enabled the time-dependent following of metabolic changes during naturally occurring developmental stages of plants and crops, e.g. for Arabidopsis (Fait et al., 2006), strawberries (Fait et al., 2008) and potatoes (Davies, 2007).

The germination of crops represents an important stage in the course of plant development. It is characterized by a combination of various catabolic and anabolic processes. Distinct and germination time-dependent alterations in metabolite levels are to be expected and GC-based metabolite profiling was shown to be a suitable tool to provide a comprehensive picture of these changes. Studies on crops have been conducted to follow the dynamic changing metabolic phenotype during the germination of rice (Shu et al., 2008) and the process-related germination of barley, i.g. in the course of the malting process (Frank et al., 2011).

The aim of the present work was to investigate the suitability of GC-based metabolite profiling for an unbiased analysis of sprouting mung beans. Mung beans (*Vigna radiata*) belong to the legume family and represent an excellent source of protein (El-Adawy, 1996; Fan and Sosulski, 1974; Thompson et al., 1976). Seeds and sprouts of mung beans are widespread foodstuffs in China, India, Bangladesh and South East Asia (Fery, 2002). In the recent years, mung bean sprouts also gained increased popularity in the Western countries.

Sprouting of mung beans is considered to improve their nutritional quality (El-Adawy et al., 2003). Targeted analyses have demonstrated that the germination of mung beans is accompanied by a spectrum of significant changes in metabolite contents (Savage, 1990; Mubarak, 2005; Abdel-Rahman et al., 2007). However, no metabolite profiling-based investigation in mung beans has been described yet. Therefore, a GC-based metabolite profiling approach should be developed for the extraction, detection and identification of a broad spectrum of polar and non-polar low molecular weight compounds in mung beans. The time-dependent metabolic changes during the sprouting process should be followed considering different impact factors on the metabolic phenotype of the final sprouts. Multivariate and univariate statistical tools should be applied for the investigations of the influence of genotype, growing environment, incubation temperature, illumination and industrial germination conditions on the metabolite profiles of sprouting mung beans. Finally, metabolic changes during the storage of mung bean sprouts at different temperatures should be assessed.

2 BACKGROUND

2.1 MUNG BEANS

2.1.1 Agriculture

The mung bean (*Vigna radiata* (L.) Wilczek) is one of the most important grain legumes in Asia. Mung beans are also known as “mung”, “moong” “greengram” (in India) and “mungo” (at the Philippines). They have a close genetic relationship to the Indian urdbean (also known as blackgram) (Poehlman, 1991) which is similar in growth behaviour and utilization as food and food ingredient.

Mung beans are cultivated most extensively in the India-Burma-Thailand region of Asia. In addition, they are also grown in South America, Africa, Iran, Pakistan, Vietnam, China, the Philippines, Korea, Indonesia, Sri Lanka and adjacent countries and islands in the South Pacific region (Sangsiri et al., 2005). There is no major mungbean production area above 40° North latitude (Morton et al., 1982). The worldwide production amount accounts for approximately 3.1 million tons / year, and the world´s major producers and exporters of mung beans are India, China, Vietnam, Myanmar and Thailand (AVRDC, 2010). In Thailand, 50,000 – 100,000 tons are annually produced on areas of more than 300,000 ha. (AVRDC, 2010).

The mung bean is a short-day, warm-season crop, grown mainly in semiarid to subhumid lowland tropics and subtropics with 600 to 1,000 mm annual rainfall, 20 to 40 °C mean temperature during the period of planting, and elevations not exceeding 1,800 to 2,000 m (Morton et al., 1982). Farming management practices for mung beans are similar to those for soybeans (Piara Singh et al., 2006). Mung beans can be planted during three seasons of the year, i.e. the early rainy season, the late rainy season and the dry season. The latter period is preferred because of lower pest pollution and a higher production quantity (Titapiwatanakun, 1990). Major climatic factors affecting the cultivation of mung beans are solar radiation, temperature, photoperiod and precipitation. Conditions such as windstroms and hail can be locally destructive, and high humidity may foster development of foliage diseases (Morton et al., 1982).

Planted in early June, seeds will begin to flower within 50 to 60 days, and then continue flowering for a few weeks (Figure 1). The crop is usually ready to be harvested in early to mid-September. The color of mung bean seeds ranges from green to brown (some tropical varieties are yellow), and they are about half the diameter of soybean seeds. Mung bean plants are about 60 to 80 cm tall. Pods are 7 to 10 cm long, each having 10 to 15 seeds. There are several pods clustered at a leaf axil, with typically 30 to 40 pods per plant (Morton et al., 1982). The pods turn darker in color as they mature.

Figure 1: Planting (A, Pulse foods, 2009), flowering (B, Kovanpinarioo, 2008) and harvesting (C, FAO/IEAE, 2010) of mung beans.

In Thailand, mung beans are ideally planted in early May, although planting dates until the middle of the month are appropriate. For seeding, a rate of 17 kilograms per hectare is recommended for wide rows and up to 22 kilograms per hectare should be planted if narrow rows are used. Mung bean plant populations in wide rows are similar to soybeans, about 4 to 8 plants per 30 cm of row (Shanmugasundaram et al., 2009). Field trials in Thailand have been done with 76 cm row spacings to allow row crop cultivation for weed control. Mung bean seeds are planted more shallow than soybeans with a depth of 2.5 cm in most soils. However, the depth can be adjusted up to 4 cm in sandy soils (Shanmugasundaram, 2009).

As a legume that fixes its own nitrogen, mung beans do not need nitrogen fertilizer. Potassium and phosphorous needs have not been studied for mung beans (Poehlman, 1991). However, amounts used for soil tests from the fertilization of soybeans are recommended to be also appropriate for mung beans (Shanmugasundaram, 2009). The optimum soil pH for the growth of mung beans is close to pH 7 (Oplinger et al., 1990).

Within the last 10 years, efforts have been made to develop new mung bean cultivars by means of conventional, biotechnological and mutation breeding (Ngampongsai et al., 2008). Induced mutation breeding using physical and chemical mutagens represents a method to create genetic variation resulting in new varieties with enhanced characteristics. The application of radiation and chemical mutation in mung bean breeding for various aspects were undertaken. The selection and development of mutants into recommended varieties for the farmers have been successfully made in many countries (Wongpiyasatid *et al*., 1998 and 1999). Sandhu and Saxena (2003) studied different mung bean mutant lines and found high variations in yield per plant and nutritional quality, especially for contents of protein, methionine, tryptophan, sulphur, phenol and total sugars. In Thailand, mutation breeding is being used to improve mung bean varieties regarding a higher yield and an improved resistance to diseases compared to previously used varieties. A comparison of different lines in 1997 revealed that most mung beans had agronomic characteristics not statistically different from each other. To date, some lines gave higher yields with less powdery mildew infections than the comparators (Ngampongsai et al., 2008).

2.1.2 Properties and utilization

On average, mung beans consist of 44g available carbohydrates, 24g protein, 16g total dietary fibre, 3g minerals and 1.3g fat per 100g edible portion (Kirchhoff, 2008). Mung beans are rich in raffinose, stachyose, verbascose and cellulose. The phytic acid content is less than 1% (Kirchhoff, 2008). Mung bean sprouts contain 1.8g available carbohydrates, 3.2g protein, 1.2g total dietary fibre, 0.4g minerals and 0.4g fat per 100g edible portion. Soybean sprouts consist of 4.7g available carbohydrates, 6.3g protein, 2.4g total dietary fibre, 0.8g minerals and 1g fat per 100g edible portion, but phytic content is higher than in mung beans (Kirchhoff, 2008).

Mung beans are mainly grown for their protein-rich edible seeds which can be easily digested by humans when consumed as food (Poehlman, 1991). Like most legumes, mung beans are relatively high in protein, around 25% of the seed by weight. The amino acid profile of mungbeans is very similar to other beans like fafa beans (*Vicia faba* L.) and cowpea (*Vigna unguiculata* L.) (Kirchhoff, 2008).

Mung beans are used for several food products, both as whole seeds and in processed form. Commonly known mung bean-derived products are noodles, bread, curry, soups and desserts (Figure 2).

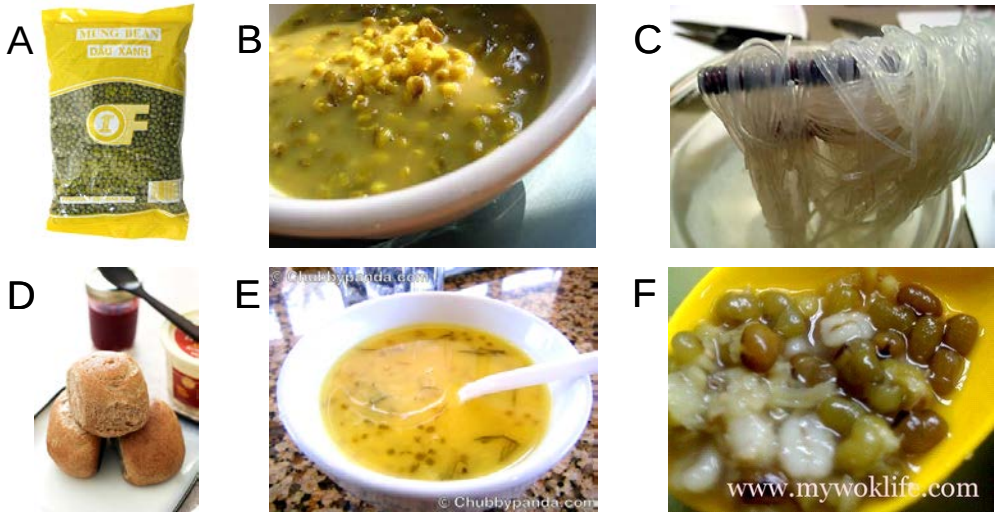


Figure 2: Commercially available mung bean seeds (A, www.steenbergs.co.uk) and food products from mung beans (B-F). B, soup (www.chubbypanda.com); C, noodles; D, bread (www.chowtimes.com); E, curry (www.chubbypanda.com); F, sweets (www.123rf.com).

2.1.3 Mung bean sprouts

2.1.3.1 Sprouting process

The process of sprouting is deliberately employed in the production of foods from legumes. Mung bean sprouts represent an outstanding example for germinated legumes represents they are widely spread foods in China, India, Bangladesh and South East Asia (Fery, 2002). The production of sprouts from mung beans is a simple germination process that requires neither sunlight nor soil and is not limited to seasonal impacts. A simple procedure to obtain the sprouts as described by Tsou et al. (1985) is shown in Figure 3.

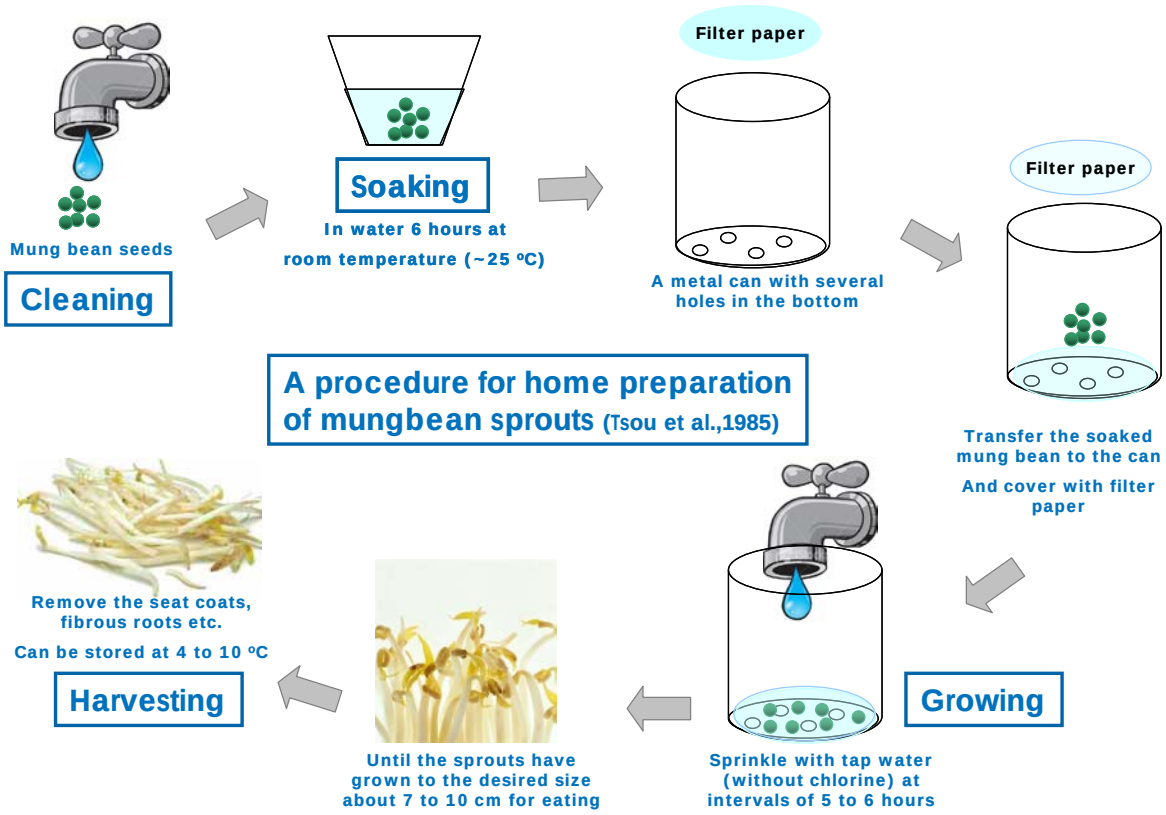


Figure 3: Sprouting process for mung beans according to Tsou et al., 1985.

Mung bean seeds exhibiting less than 15% moisture content can be safely stored under cool and dry conditions before they are subjected to the sprouting process without a loss of germination ability (Shanmugasundaram, 2008). Prior to germination, mung beans should be washed in fresh water to allow the empty and broken seeds to float. Floating seeds and debris can then be removed. After the washing step, the mung beans are usually soaked in tap water at room temperature for 6 to 12 hours (Lal and Shanmugasundaram, 1971). For an “in-house” germination, after soaking, mung beans are placed between filter papers into a container and watered regularly every 5 to 6 hours. After a total incubation time of 72 hours, the sprouts can be harvested.

The home sprouting in Thailand is a simple to handle task. The sprouting process is completed in 2 to 4 days. The sprout production is extremely inexpensive, requiring only mungbean seeds, sprouting containers and water as inputs. Mung beans are soaked in room-tempered water for 6-12 hours. Soaked mung beans are put into a steamer pot or container that can strain water. The container is kept in the dark at an

ambient room temperature and watered and rinsed two times a day (morning and evening).

The procedure takes about 3-5 days before the sprouts are ready to eat (depends on summer or winter climate). After obtaining the mature sprouts, the sprouts are cleaned with running water. Green beans which were not sprouted are manually removed. In addition, the roots are also removed before consumption. After the sprouting procedure, sprouts are washed about 10-times in water mixed with a few table spoons white vinegar to keep sprouts white and fresh. The sprouts can be stored in an air-tight container in a refrigerator for up to one week.

For the commercial sprout production of mung beans, after soaking process all sprouting operations are performed in a sheltered place to protect the sprouts from light. Darkness ensures bright white and long mung bean sprouts. Moderate temperatures (25-30ºC) and high humidity (85-90%) in the sprouting room help to produce good quality crispy sprouts (Shanmugasundaram, 2008).

Sprouting mung beans increase to a length of 2 to 3 cm after 1 to 2 days (mini-mungbean) and reach a length of 8 to 9 cm after 3 to 4 days. Consumer preferences determine the size of sprouts produced.

Sprouts of marketable size are transferred from the sprouting containers to drums, troughs or buckets for washing. The sprouts are washed and packed during the early morning to avoid spoilage by high temperatures after sunrise. Broken roots, sprout pieces and other debris are removed by washing with fresh cold water 3-4 times. In addition, seed coats still attached to the sprouts can be removed by the washing step. Sprouts are removed from the drum by using a basket or sieve (Lal and Shanmugasundaram, 1971).

2.1.3.2 Properties and utilization

Germination is considered to improve the nutritional quality of mung beans (El-Adawy et al., 2003). Targeted analyses have demonstrated that the germination of mung beans is accompanied by a spectrum of significant changes in metabolite contents (Savage, 1990; Mubarak, 2005; Abdel-Rahman et al., 2007). For example, decreased triglyceride contents (Abdel-Rahman et al., 2007) and increased levels of free amino acids (Kavas and El, 1991) have been reported in mung bean sprouts.

The major minerals are iron and zinc; nicotinamide and pantothenic acid are the main vitamins found in mung bean sprouts (Kirchhoff, 2008). Mung bean sprouts are a good source of digestible energy, bioavailable vitamins, minerals, amino acids, proteins and phytochemicals which are necessary for a germinating plant to grow (Spring, 2002). Further, these nutrients are essential for human health.

Mung beans are the most common and easily available type of sprouts. They are used to a large extent in Asian cuisines and have a sweet, nutty flavor and crisp texture. Uses include adding to cooked dishes at the end of the process. The thick-stemmed sprouts can withstand several minutes' worth of cooking. Mung beans sprouts can be stir-fried or cooked in traditional Chinese dishes like chicken chow mein and chop suey, or eaten raw in salads and sandwiches (Shanmugasungdaram, 2008). Soybeans have a more pronounced bean-like flavor and are often preferred cooked. Soybeans are harder to digest when raw. For this reason, cooking them in stews and soups, or adding to a stir-fry is more appropriate (Shanmugasungdaram, 2008). Alfalfa bean sprouts have a grass-like appearance with small purple flowers whereas the mung bean sprouts are cream-colored with a white shoot. As a sprawling field crop belonging to the pea family, alfalfa is harvested just like grass or hay.

Bean sprouts are mostly consumed by humans, whereas alfalfa is mainly given to the livestock. Bean sprouts, which are very much used in Asian cuisine, are consumed as stir fries, soups and salads. Alfalfa is mainly seen as a dehydrated supplement in herbal pills and teas. Unlike alfalfa, bean sprouts should be eaten within four to five days. Regarding the composition, alfalfa sprouts and bean sprouts are different. Although both alfalfa and bean sprouts are free of cholesterol and contain only few calories, their nutritional values differ: one cup of alfalfa contains 1.32g of protein whereas one cup of bean sprout contains 3g of protein. However, both alfalfa and mung bean sprouts are high in vitamin A, E, B, C, amino acids and antioxidants. In addition, alfalfa sprouts contain higher levels of phytochemicals. The biggest alfalfa producers are the USA and the biggest producer of mung beans is Asia (USDA/ARS, 2000).

Sprouted mung beans can be eaten raw like many other legumes. The difference compared to other beans is that mung beans are quite easy to digest without causing flatulence reactions. Cooked mung beans and sprouts are delicious with spices and seasonings. They can be cooked as soup and casserole. Bean sprouts are a good

source of vitamin C and make a nutritious addition to sandwiches and salads (www.wisegeek.com).

2.1.3.3 Contamination hazards

Sprouts should be cooled immediately after the germination process and the temperature should be held at 0 °C. Vacuum-cooling, hydro-cooling, and forced-air cooling are common methods. Sprouts are highly perishable and most last 5 to 10 days at 0 °C (Bennik, 1997). Sprouts stored at 0, 2.5, 5 or 10 °C reach the lower limit of marketability after 8.5, 5.5, 4.5 and 2.5 days, respectively (Lipton et al., 1981). Alfalfa and radish sprouts stored at 0 °C with > 95% relative humidity had a shelf-life of 5 to 7 days (Cantwell, 1997). The shelf-life of mung bean sprouts can be increased by storage under modified atmosphere in which O_2 is reduced and CO_2 is increased (Varoquaux et al., 1996). For instance, sprouts can be held for 4 to 5 days at 8 °C in packages containing 5% O_2 + 15% CO_2. Darkening of sprouts is reduced and development of sliminess is delayed.

Sprouts which are not grown and processed under hygienic conditions or stored under inadequate conditions can cause food poisonings through pathogenic microorganisms such as *Salmonella* or *E. coli* O157:H7 (Schrader, 2002; Mohle-Boetani et al., 2009). In addition, intact seeds may be contaminated with microbes before they were subjected to the sprouting process. *Salmonella* or *E. coli* could be harbored in bird droppings, in manure applied to fields as fertilizer, in contaminated water that is used to irrigate fields or in dirt left over in improperly cleaned seed-sorting machinery. The pathogens might also live in droppings of rodents that eat seeds stored in bags, bins or silos (Schrader, 2002).

2.1.3.4 Economics

Domestic utilization of mung beans in Thailand was 87.39 thousand tons or 47% of its total production. The export volume and value of mung beans from Thailand to major countries between 1981 and 2005 was approximately 111.4 thousand tons, with China receiving 36.5%, Taiwan 13.6%, India 12.7%, Hong Kong 4.9%, Singapore 3.8%, USA 3.1% and other countries 23.2% (Office of Agricultural Economics (OAE) Mininstry of Agriculture and Co-operatives, (MOAC), Bangkok, Thailand, 2006).

Most human consumption of mung beans is in the form of bean sprouts, which is popular in many typical Thai dishes. It was estimated by the traders that the annual per capita consumption of bean sprouts was about 0.7 to 1.0 kg. This would mean that in 1987, the total of mung beans consumed in the form of bean sprouts was about 37.0-52.9 thousand tons. Mung beans are also consumed as a dessert after either boiling the whole bean or boiling the dehulled half-bean with sugar. Although figures for the amount of mung beans consumed as a dessert are not available, the estimation made by the Department of Agricultural Extension on other usage of mungbean at 10 thousand tons per year, might be regarded as an approximation of annual mung bean consumption as dessert (Office of Agricultural Economics (OAE) Ministry of Agriculture and Co-operatives, (MOAC), Bangkok, Thailand, 2006).

2.2 PLANT METABOLOMICS

2.2.1 Historical overview

In clinical research, chromatography-based approaches for the investigation of metabolic patterns and profiles have been developed already in the 1960s and 1970s (Dalgliesh et al., 1966; Horning and Horning, 1971, Pauling et al., 1971). However, it took another 20 years until the first milestone was set in the field of plant metabolomics. Sauter et al. (1991) developed at BASF Ludwigshafen, Germany, an analytical procedure for the metabolic profiling of barley plants based on extraction, silylation and capillary gas chromatography. Barley seedlings were treated with various herbicides at sublethal doses and the profiles from the ethanolic extracts of the shoots were compared to the untreated plants. This new diagnostic technique in plant science allowed the detection of 100-200 peaks in a single chromatogram. On the basis of this approach, scientists from the Max-Planck-Institute for Molecular Plant Physiology in Golm, Germany, developed around the turn of the millennium a comprehensive GC-MS-based metabolite profiling methodology for the simultaneous determination of a broad range of metabolites in *Arabidopsis thaliana* leaves (Fiehn et al., 2000a, 2000b) and potato tubers (Roessner et al., 2000; Roessner et al., 2001). The qualitative and quantitative coverage of more than 150 compounds enabled the phenotyping of differently expressed plant systems by means of univariate and multivariate data assessments. Additionally, these studies paved the way for metabolomics as a tool for plant and crop functional genomics. Following this pioneer work, in the beginning of the 2000s, several groups / institutions / programs emerged (Sumner et al., 2003) which developed and applied metabolomics-based methods for the investigation of plants and plant-derived crops like *Medicago* (Huhman and Sumner, 2002), *Oryza sativa* (Frenzel et al., 2002) and *Solanum lycopersicum* (Hall et al., 2002).

The increasing scientific interest in plant profiling techniques led to the first international congress on plant metabolomics held by Plant Research International in Wageningen, The Netherlands, in 2002 with the aim “of bringing together those players who are already active in this field and those who soon plan to be” (Hall et al., 2002). Only one year later, Harrigan and Goodacre edited the first book on

metabolomics entitled "*Metabolic profiling: its role in biomarker discovery and gene function analysis*", in which an overview on the potential of metabolome research was presented (Harrigan and Goodacre, 2003). Having raised attention, the topic of metabolomics found its way into several special issues of high-impact scientific journals and the appreciation of metabolome research culminated in the specific journal called *Metabolomics* introduced in 2005 (http://www.springer.com).

Due to progress in technologies and computational advances in the 2000s, plant metabolomics approaches have been established on various instrumental platforms and expanded to high throughput and large scale plant systems biology (Weckwerth, 2003; Fernie et al., 2004; Kopka et al., 2004). At the same time, researchers claimed the suitability of unbiased metabolomics as an additional tool for the safety assessment of genetically modified plants and crops because of its potential to increase the probability to detect unintended effects (Kuiper et al., 2003; König et al., 2004). The origin of those considerations dates back to 2000 when Noteborn et al. (2000) used liquid chromatography coupled to NMR for a chemical fingerprinting of transgenic tomatoes. In the meantime, several metabolomics studies have been conducted for the assessment of genetically modified crops (Shintu et al., 2009; Ricroch et al., 2011; Frank et al., 2012a).

2.2.2 Definitions

The rapidly growing spectrum of different analytical and instrumental methods used in the metabolomics community made it necessary to introduce some definitions regarding the metabolome analysis. A summarizing overview on the different types of metabolomics-based approaches has been given by Saito and Matsuda (2010). In analogy to the already established terms genome and proteome, Oliver et al. (1998) established the term metabolome as the "entity of all metabolites in a biological system" in the field of yeast functional genomics. Trethewey et al. (1999) gave a first definition on metabolic profiling as a method that comprises a wide range of compounds in a single measurement. However, it was also noted that metabolic profiling is associated with a lower degree of precision compared to targeted analytical methods. The so-called metabolic or metabolite profiling can be considered as one of the most pragmatic approaches presently applied. It aims at the detection, identification and quantification of a broad spectrum of compounds in a single sample

in an effective and reproducible way to provide a deeper insight into the complex biological system (Fiehn et al., 2000b; Fiehn, 2002; Kopka et al., 2004). By definition, “metabolite profiling” represents the analysis of selected compounds from the same chemical compound classes or compounds linked by known metabolic relationships (Allwood et al., 2008). In contrast, “metabolic fingerprinting” represents a rapid screening method for biological samples without a major pretreatment of the plant material to be analyzed. In order to screen a large number of these samples, e.g. in plant breeding programs, it might not be necessary to determine the individual level of every metabolite. Instead, a rapid classification of samples according to their biological relevance might be sufficient (Allwood et al., 2008).

2.2.3 Techniques

In recent years, multiple-platform profiling and fingerprinting techniques have been developed and applied to comprehensive analyses of plant / crop genotypes and phenotypes. Established platforms are gas chromatography-mass spectrometry (GC-MS), (high and ultra performance) liquid chromatography-mass spectrometry (HPLC-MS, UPLC-MS), direct infusion-mass spectrometry (DI-MS), capillary electrophoresis-mass spectrometry (CE-MS), Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), nuclear magnetic resonance spectroscopy (NMR), Fourier transform infrared-spectroscopy (FT-IR) as well as hyphenated techniques (Dunn and Ellis, 2005; Allwood et at., 2008, 2009, 2010, 2011; Hagel and Facchini, 2008; Harada and Fukusaki, 2009; Hall, 2011; Ramautar et al., 2011). Coupling of GC or LC and mass spectrometry have proven to be the most applied methodologies within the scope of plant metabolite profiling (Trethewey, 2006; Steinhauser and Kopka, 2007; Moritz and Johansson, 2008; Bedair and Sumner, 2008). Newer GC-time-of-flight (Tof)-MS systems combine a high separation efficiency and resolution of capillary GC with the high sensitivity of mass-selective detection making it attractive in GC-based metabolome analysis. However, owing to inherent features of the approach, e.g. choice of extraction solvents, derivatization steps or volatilities of derivatives, it has to be considered that the type of metabolites covered is to some extent pre-determined and not fully “unbiased”. Therefore, and based on the assumption that single plants may contain potentially up to 20 000 metabolites (Fernie et al., 2004), the use of a single GC technique still has its limitations.

Therefore, the application of multi-dimensional GC or multiple-platform crossing techniques can help to improve the detection and identification of plant metabolites for qualitative and quantitative metabolite profiling of plant systems. A promising approach is represented by a two-dimensional GC combined with time-of-flight mass spectrometry (GCxGC-Tof-MS). This strategy allowed, for example, the detection of more than 1400 peaks in a single chromatogram obtained from a plant leaf extract (Pierce et al., 2006). In addition to GC, LC-based investigation of metabolites is an aspiring field in metabolomics (Theodoridis et al., 2008). In contrast to GC analysis, fewer sample preparation steps (e.g. derivatization) are necessary. Moreover, LC analyses can be adapted to a wider array of substances, including a range of secondary plant metabolites such as alkaloids, flavonoids, glucosinolates, isoprenes and saponins (Fernie et al., 2004). However, this technique has some drawbacks in the chromatographic performance compared to GC. The use of UPLC might overcome these drawbacks by increasing the chromatographic resolution and additionally allowing a more rapid analysis in plant metabolomics studies (Wilson et al., 2005; Moritz and Johansson, 2008; Grata et al., 2009; Allwood and Goodacre, 2010; Eugster et al., 2011). A comprehensive overview on advantages and disadvantages of GC and LC-based mass spectrometric methods is given by Hall (2011).

For plant metabolic fingerprinting, non-destructive spectroscopic approaches like ^{1}H-NMR and ^{13}C-NMR (Krishnan et al., 2005; Colquhoun, 2007; Schripsema, 2010; Kim et al. 2010, 2011; Leiss et al., 2011; Palomino-Schätzlein et al., 2011) and FT-IR (Gidman et al., 2003; Johnson et al., 2004) are being used without a chromatographic separation of individual compounds. Nevertheless, NMR has proven to be a suitable instrumental platform for the coverage of both abundant primary metabolites (e.g. sugars, amino acids) as well as secondary plant metabolites (e.g. flavonoids, alkaloids) (Leiss et al., 2011). An overview on NMR-based plant metabolomics studies is given by Verpoorte et al. (2007) and Kim et al. (2011). An advantage of NMR is that its signals are proportional to the metabolite molar concentrations which allows the direct comparison of concentrations of all metabolites without the preparation of calibration curves (Kim et al., 2010). On the other hand, a major drawback of NMR is its relatively low sensitivity compared to MS-based methods. Therefore, hyphenated techniques that couple chromatography to

mass spectrometry or to NMR are considered as powerful combinations, in particular with respect to compound identification (Bino et al., 2004).

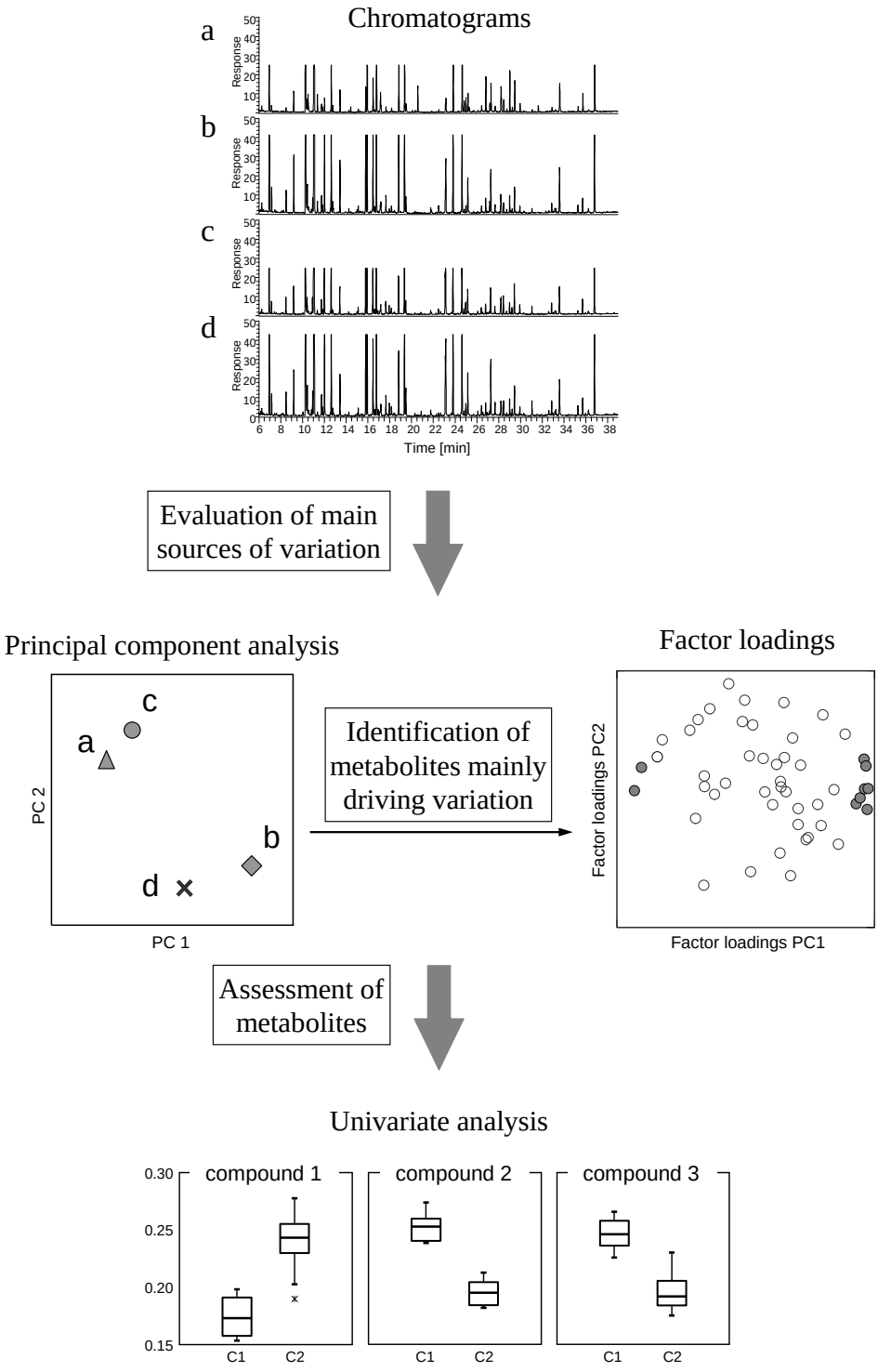
A method which is coming increasingly in the focus of interest is FT-ICR-MS. It is characterized by an extreme mass-resolving power, detection sensitivity and mass accuracy (Hagel and Facchini, 2008). FT-ICR-MS has been applied to metabolomics by plant biologists and used to investigate developmental changes, stress-responses, varietal differences, phenotyping and impact of genetic modification. A good overview of the potential and use of FT-ICR-MS in plant and crop metabolomics has been given by Daskalchuk et al. (2006) and Ohta et al. (2007). In addition, capillary electrophoresis mass spectrometry (CE-MS) represents an interesting method for the metabolite profiling of polar and charged compounds. It rapidly separates such compounds on the basis of their m/z ratio without a major sample pretreatment. A good overview on CE-MS applications in plant metabolomics is given by Ramautar et al. (2011).

In addition to the mentioned platforms, there is an increasing demand to link metabolomics to other omics-techniques, i.e. genomics, transcriptomics and proteomics in order to strengthen the power of plant functional genomics.

2.2.4 Metabolite Profiling Workflow

The application of metabolite profiling to broad arrays of samples results in a huge amount of metabolite-related data. Therefore, the traditional data analysis based on “value-by-value” comparisons is not satisfactory. For metabolite profiling data it is more appropriate to start with a multivariate analysis approach for the overall determination of variation in a dataset followed by univariate analysis of metabolites shown to be relevant in the foregoing multivariate approach.

Multivariate statistical analytical methods such as principal component analysis (PCA), hierarchical cluster analysis (HCA) and self-organizing maps (SOMs) proved to be useful tools in the analysis and evaluation of metabolite profiling data (Sumner et al., 2003; Roessner et al., 2001; Pierce et al., 2006). Principal component analysis enables the rapid differentiation of samples based on their metabolite profiles by visualizing the data as dots in a two-dimensional plot (Figure 4).



Metabolite	C1	CI1	C2	CI2
compound 1	0.81	0.072	0.53	0.032
compound 2	0.61	0.047	0.42	0.040
compound 3	0.04	0.003	0.03	0.002
compound 4	0.06	0.034	0.15	0.051

Figure 4: Workflow of capillary gas chromatography-based metabolite profiling.

The investigation of the major sources of variation is followed by a substantiation of differences. An efficient way to identify the drivers of variation is to examine the factor loadings as determined by PCA. For example, if a clear separation of samples is revealed on the first principal component, the main contributors to this effect will exhibit high absolute loading factors in PC1 (Figure 4). In a subsequent step, the signals of target compounds can be evaluated using univariate statistical approaches such as Student´s t-test, analysis of variance (ANOVA) or in form of box plots.
A major future challenge for metabolomics will be the implementation of reporting standards as suggested by the Metabolomics Standards Initiative (Fiehn et al., 2007). This would ensure the comparability of metabolite profiling data to be used for crop metabolite databases.

2.2.5 Metabolite profiling of plant-derived foods

During the past years, numerous studies have been conducted to the investigation of quality and safety of plants and crops from various families of the plant kingdom. An overview on potential applications of metabolite profiling to plant-derived foods is given in Figure 5.
Unbiased profiling methods using numerous instrumental platforms have been applied amongst others to assess the naturally occurring metabolic diversity and genetic variation in crops (Harrigan et al., 2007; Davies et al., 2010; Maltese and Verpoorte, 2010), to investigate the plant metabolic response to stress induction, e.g. through microbial inoculation, heavy metals or drought (Shulaev et al., 2008), to follow metabolic changes in the course of plant developmental stages (Shu et al., 2008), to discover plant metabolic biomarkers for phenotype prediction (Steinfath et al., 2010), to annotate plant gene functions (Tohge and Fernie, 2010), to elucidate plant metabolic pathways (Dixon et al., 2006), to improve the nutritional quality of plant-derived crops (Fernie and Schauer, 2009; Hall et al., 2008) and to assess potential unintended effects in genetically modified foods (Hoekenga, 2008; García-Canas et al., 2011; Ricroch et al., 2011) even in the light of environmental influences (Shintu et al., 2009; Frank et al., 2012a).

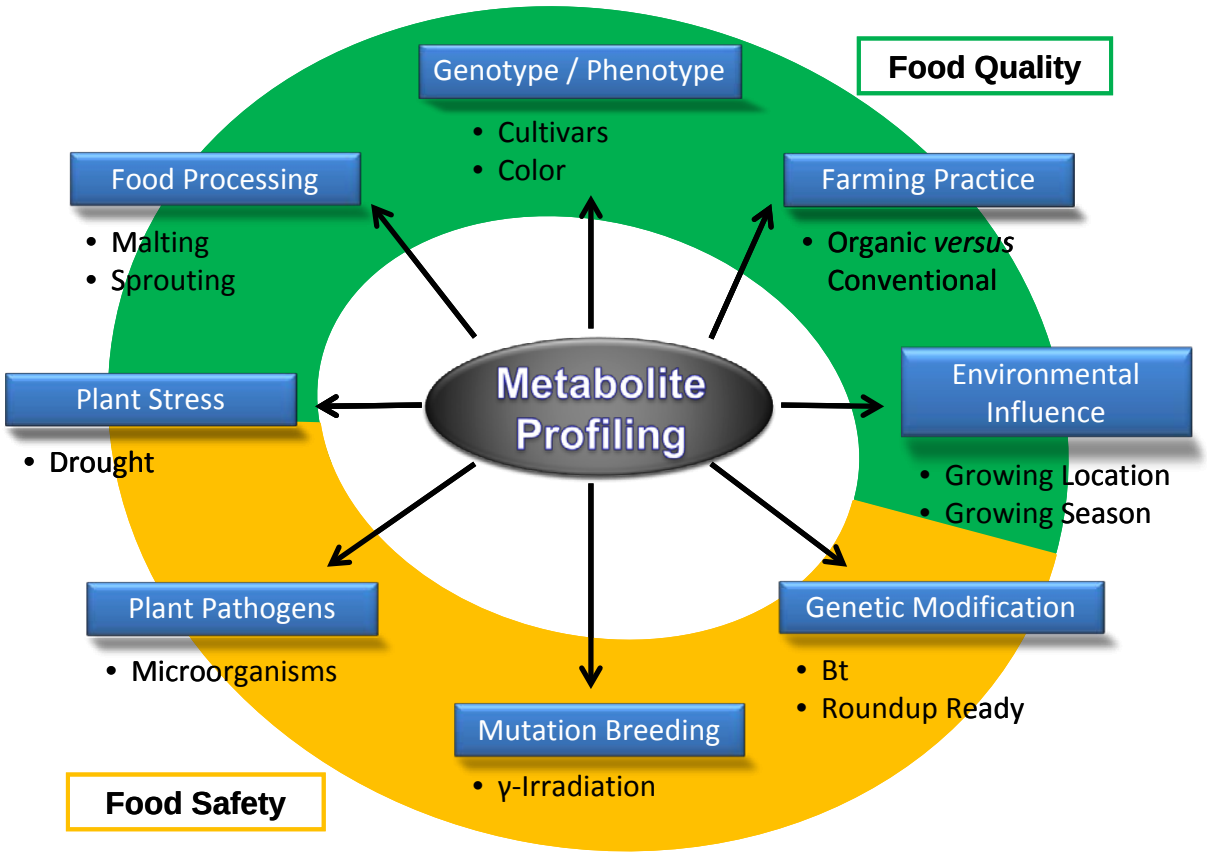


Figure 5: Applications of metabolite profiling to the assessment of plant-derived foods.

2.2.5.1 Crop quality

Untargeted metabolite profiling as an approach for the simultaneous detection, identification and quantification of a broad spectrum of polar and non-polar constituents represents a suitable tool for the investigation of crop metabolite phenotypes. Coverage of different impact factors on the crop metabolite profiles allow to assess the overall metabolic variation and thus provides comprehensive data for breeders to produce high-quality foods. As example, metabolite profiling has been applied in order to improve the productivity and nutritional quality of tomato fruits (Iijima and Aoki, 2009).

2.2.5.2 Crop safety

For the detection of potential unintended effects in novel foods, targeted and non-targeted approaches are being discussed (Cellini et al., 2004). On the food

composition level, targeted methods are used for the analysis of specific compounds, e.g. macronutrients, micronutrients and toxins (EFSA, 2011). However, those targeted approaches have limitations owing to their biased character. That means, only known compounds can be analyzed with respect to these methods. Therefore, unbiased metabolite profiling approaches are considered as additional tools for the safety assessment of GM-derived crops (Kuiper et al., 2003; Davies, 2009; Shintu et al., 2009). Metabolite profiling-based comparative investigations of crops grown under different environmental conditions enable consistent differences to be searched for in order to distinguish between natural variability and changes induced by different treatments such as genetic modification. In combination with an appropriate statistical data analysis, metabolite profiling therefore increases the probability of detecting effects not intended by the genetic modification and thus contributes to the safety assessment of such crops. Comprehensive overviews on metabolite profiling-based comparative studies of genetically modified and non-GM crops including barley, rice, wheat and maize are given in literature (Harrigan et al., 2010; Ricroch et al., 2011).

2.2.5.3 Food processing

Metabolomics approaches are being employed to follow developmental changes in crops. An important stage in plant biology is represented by germination. Metabolic changes within this stage have been investigated by means of metabolite profiling in various crops including rice (Shu et al., 2008) and potatoes (Roessner et al., 2001). In addition to the investigations of solely “natural” biological changes in crops during their life-cycle, metabolomics has become a rapidly emerging analytical approach in food science including food processing (Cevallos-Cevallos et al., 2009). As example, malting - a process-related germination - of barley as one of the most important applications in food technology has been investigated by a GC-MS-based metabolite profiling (Frank et al., 2011). Due to the simultaneous coverage of a comprehensive set of metabolic data, metabolite profiling may assist in providing valuable information for further improvements of the quality of processed foods.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

The following chemicals were used:

Acetone	VWR International, Darmstadt
Acetonitril (LiChrosolv®, gradient grade)	Merck, Darmstadt
Argon (purity: 4.6)	Air Liquide, Maisach
4-Chloro-L-phenylalanine (puriss.)	Fluka, Taufkirchen
5α-Cholestane-3β-ol	Fluka, Taufkirchen
Dichloromethane (puriss.)	Fluka, Taufkirchen
Ethanol	Merck, Darmstadt
Formic acid	Fluka, Taufkirchen
Helium (purity: 5.0)	Air Liquide, Maisach
Hexadecane (puriss. p.a.)	Fluka, Taufkirchen
Hexan (p.a.)	VWR International, Darmstadt
Hydrogen (purity: 5.0)	Air Liquide, Maisach
Hydroxylammoniumchloride (for Synthesis)	Merck, Darmstadt
Methanol (LiChrosolv®, gradient grade)	Merck, Darmstadt
Methyl-*tert*-butylether (Driveron S)	Oxeno Olefinchemie, Marl
N-Methyl-*N*-trimethylsilyl-trifluoroacetamide	Fluka, Taufkirchen
N-Trimethylsilylimidazole	Fluka, Taufkirchen
Natriummethoxide Solution (30% in Methanol)	Fluka, Taufkirchen
Natriumsulfat (puriss. p.a.)	VWR International, Darmstadt
Octatriacontane (puriss. p.a.)	Fluka, Taufkirchen
Petroleum ether	Merck, Darmstadt
Phenyl-β-D-glucopyranoside	Fluka, Taufkirchen
Phytic acid dodecasodium salt hydrate	Sigma-Aldrich, Steinheim
Pyridine (puriss. p.a.)	Fluka, Taufkirchen
Hydrochloric acid, 37%	Fluka, Taufkirchen
Nitrogen (purity: 5.0)	Air Liquide, Maisach

Sulfuric acid	Fluka, Taufkirchen
Tetrabutylammoniumhydroxide (puriss. p.a.)	Fluka, Taufkirchen
Tetracosane (puriss. p.a.)	Fluka, Taufkirchen
Toluol	VWR International, Darmstadt
Triacontane (puriss. p.a.)	Fluka, Taufkirchen
Undecane	Fluka, Taufkirchen

Methyl-*tert*-butylether was distilled before usage. All other reagents and solvents were of analytical grade. Authentic reference compounds used for identification and quantification were obtained from Fluka, Merck, Sigma-Aldrich, VWR International and Supelco.

3.1.2 Mung bean materials

Intact mung beans were obtained from an industrial sprout producer (Deiters & Florin GmbH, Hamburg, Germany), from the Chai Nat Field Crops Research Center (Kampang Saen 2 and Chai Nat 72; Chai Nat, Thailand) and were purchased in a local health-food store (Freising, Germany).

3.1.3 Equipment

The following equipments were used:

ACTEVap® Evaporator	Advanced Chemtech, Gießen
Analytical balance Sartorius research R300S	Sartorius, Göttingen
Cyclon mill Cyclotex 1093 (0.5 mm sieve)	Foxx Tecator, Hamburg
Freeze-drying apparatus Christ 1-4 LSC	Martin Christ, Osterode
Glass syringes (10 µL, 50 µL, 250µL)	BGB Analytik,
Silica gel cartridges LiChrolut® Si 60, 3 mL	VWR International, Darmstadt
Manifold Visiprep™	Supelco, München
Membrane filter Spartan 0.45 µm	Schleicher & Schuell, Dassel
Oil bath, consisting of :	
- Heatable magnetic stirrer RH basic	IKA Labortechnik, Staufen

- Thermo stable fluid Labothermol® S	neoLab, Heidelberg
- Glass dish	Zefa Laborservice, Harhausen
- Thermometer ETS-D4	IKA Labortechnik, Staufen
- Magnetic stirring staff	VWR International, Darmstadt
Vacuum rotary evaporator VV2001	Heidolph, Schwabach
Supersonic bath Sonorex RK 100H	Bandelin Electronic, Berlin
Vortexer VF2	IKA Labortechnik, Staufen
Water treatment apparatus Milli-Q plus	Millipore, Eschborn

3.2 METHODS

3.2.1 Metabolite profiling of mung beans

3.2.1.1 Sample processing

Method development. The mung bean seeds were cleaned and rinsed with tap water. 500g of the seeds were soaked in 5 l of tap water for 12h at 35 °C. The soaked seeds were placed between moist filter paper in petri dishes and incubated at 35 °C. Samples were taken after 0h (before soaking), 24h, 36h, 48h, 60h and 75h. Samples were immediately frozen in liquid nitrogen and ground with a cyclone mill equipped with a 500-µm sieve (Cyclotec, Foss, Germany). The flour was freeze-dried for 48 hours using a conventional freeze-drying apparatus (Alpha 1-4 LSC, Christ, Germany). Freeze-dried flour was stored until analysis at -18°C in tightly closed bottles until analysis. After four weeks a second germination experiment was performed under the same conditions.

Impact of pre-treatment and sprouting temperature. The germination of mung bean was also carried out in laboratory-scale at different sprouting temperatures. Two sample sets were incubated at 25 °C for germination after washing the mung beans with tap water and with methanol. In addition, mung beans were incubated at 35 °C after washing with tap water. For each sample set, 500 g of mung bean materials from the same batch were used for the germination process. The mung beans were soaked for 12 h and subsequently germinated between wet filter papers at different temperature conditions (25°C and 35°C). In addition to the ungerminated mung bean (0 h), samples were taken in the course of germination after 6, 12, 18, 24, 30, 36, 42, 48, 54, 60 and 75 hours. The total of 34 mung bean samples were freeze-dried, milled and stored similar to the description above.

Influence of illumination. The sprouting of mung beans was carried in an incubator. One batch was germinated completely in the dark and one batch under artificial light exposure Mung beans were washed with tap water and soaked for 12 h at 30°C. They were then placed between moist filter paper in plastic trays in an incubator for germination.

The light source for the germination under illumination was a 20 watt desk lamp, which was placed in the incubator. Average temperatures of 28°C were determined in the incubator during the sprouting progress. To prevent an overheating of the lamp, it was switched off every day for 1 hour. Germinating mung bean samples were kept permanently wet during the whole germination procedure. Samples taken after 24h, 48h and 72h were treated similar to the description above.

Industrial sprouting procedure. Before sprouting, mung bean seeds were washed and liberated from residues by means of a floating process. Mung beans were soaked in tap water for 12 h at approximately 25°C. The sprouting of mung beans was conducted in the dark in a regularly irrigated container with a base area of 1.5 x 1.0 meters at an ambient room temperature of 25°C. Samples were taken before soaking (0h) and after 24, 48 and 72 h of germination. Sprouting mung beans after 24 h and sprouts after 72 h of germination were treated three times with citric acid-containing water (pH 2.8-2.9) for 30 s each time.

3.2.1.2 Sample extraction

The freeze-dried mung bean flour (300 mg of the intact mung beans and of the mung beans incubated for 24h; 100 mg for all other time-points) was weighed into a disposable cartridge (3 ml column volume, VWR International, Germany) which was sealed with plastic frits at the top and at the bottom of the flour layer. The cartridge was subsequently connected to a vacuum manifold equipped with valves to control the flow rate. The valves were closed and the flour was soaked in 200 µl of methanol for 20 min at ambient temperature. After removal of methanol by application of vacuum (25 mbar) on top of the cartridge for 30 min, lipids were eluted by gravity flow with a total of 4 ml dichloromethane within 20 min. Subsequently, polar compounds were eluted with a total of 10 ml of a mixture of methanol and deionized water (80+20, v/v) within 40 min by application of weak vacuum at the bottom.

3.2.1.3 Preparation of standard solutions

Internal standards (tetracosane, 5α-cholestan-3ß-ol, phenyl-ß-D-glucopyranoside, *p*-chloro-L-phenylalanine) and retention time standards (undecane, hexadecane, tetracosane, triacontane, octatriacontane) were purchased from Fluka (Taufkirchen,

Germany). Acetonitrile was obtained from VWR International (Darmstadt, Germany). Other solvents and chemicals used were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (Taufkirchen, Germany).

Retention time standard 1 was prepared by adding *n*-hexane solutions of undecane (1.5 ml, 2 mg/ml), hexadecane (2.5 ml, 1.5 mg/ml), tetracosane (4 ml, 1.5 mg/ml), and triacontane (4 ml, 1.5 mg/ml) to 15 mg of octatriacontane. Retention time standard 2 was prepared by adding 1.5 ml of n-hexane and *n*-hexane solutions of hexadecane (2.5 ml, 1.5 mg/ml), tetracosane (4 ml, 1.5 mg/ml), and triacontane (4 ml, 1.5 mg/ml) to 15 mg of octatriacontane.

Internal standard solution for fraction I was identical to the retention time standard 1 (tetracosane was used as internal standard). For fraction II a solution of 5α-cholestan-3ß-ol in dichloromethane (0.3 mg/ml) was used as internal standard solution. The internal standard solution for fraction III was obtained by dissolving 40 mg of phenyl-ß-D-glucopyranoside in 50 ml of deionized water. A solution of *p*-chloro-L-phenylalanine in deionized water (0.3 mg/ml) was used as internal standard solution for fraction IV.

3.2.1.4 Fractionation and analysis of lipids

100 µl of internal standard solution I and 100 µl of internal standard solution II were added to 4 ml of the lipid extract. After evaporation to dryness under vacuum by means of an ACTEVap evaporator (40°C), the residue was redissolved in 500 µl of methyl-*tert*-butylether (MTBE), 300 µl of dry methanol and 50 µl of sodium methylate (5.4 M in methanol). After reaction for 90 min at room temperature, 1 ml of dichloromethane and 2 ml of aqueous 0.35 M hydrochloric acid were added and the mixture vigorously shaken. The upper phase was discarded and the lower phase containing the transmethylated lipids was evaporated to dryness under vacuum.

A small amount of anhydrous sodium sulfate was placed on top of a 500 mg silica gel SPE cartridge (LiChrolut, VWR International, Germany) conditioned with 2.5 ml of *n*-hexane. Transmethylated lipids were redissolved in 250 µl of dichloromethane and transferred to the SPE cartridge. The fatty acid methyl ester fraction (fraction I) was eluted with hexane/MTBE (100+2, v/v, 2 x 3 ml). Subsequently, the fraction of polar lipids (fraction II) was eluted with hexane/MTBE (70+30, v/v, 2 x 3 ml). After addition of 100 µl retention time standard 1 to fraction II, both fractions were evaporated to

dryness under vacuum (40°C). The residue of fraction I was redissolved in 300 µl of hexane, and 1 µl was injected into the gas chromatograph. After flushing the flask with argon, the residue of fraction II was redissolved in 250 µl of dry pyridine and 50 µl of N-methyl-N-trimethylsilyl-trifuoroacetamide were added. Silylation was performed in an oil bath for 15 min at 70°C. The sample was cooled to room temperature and 1 µl was analyzed by gas chromatography.

3.2.1.5 Fractionation and analysis of polar extract

After addition of internal standard solutions III (250 µl) and IV (250 µl) to the total of polar extract, 1 ml of the polar extract was evaporated to dryness under vacuum (50°C). The flask was flushed with argon and the residue redissolved in 300 µl of dry pyridine and 100 µl of trimethylsilylimidazole. Silylation was performed in an oil bath for 15 min at 70°C. The silylated sample was diluted with 300 µl of n-hexane and 300 µl of deionized water were added for selective hydrolysis of organic acids and amino acids. After shaking and phase separation, 100 µl of the upper phase containing silylated sugars and sugar alcohols (fraction III) were mixed with 50 µl of retention time standard 1 and 1 µl was injected into the gas chromatograph.

In order to obtain fraction IV (amino acids, organic acids), 2 ml of the polar extract were evaporated to dryness under vacuum (50°C). The residue was redissolved in 300 µl of a solution of hydroxylammoniumchloride in pyridine (2 mg/ml). After heating for 30 min at 70°C in an oil bath, 100 µl of N-methyl-N-trimethylsilyl-trifuoroacetamide were added while flushing the flask with argon. The sample was allowed to stand for another 15 min at 70°C. The silylated sample was diluted with 500 µl of n-hexane and 300 µl of deionized water were added. After vortexing, the upper phase containing oximated and silylated sugars was discarded. Extraction with n-hexane was repeated two times. The aqueous phase containing free organic acids and amino acids was evaporated to dryness under vacuum (50°C). The residue was redissolved in 200 µl of acetonitrile and 50 µl of N-methyl-N-trimethylsilyl-trifuoroacetamide were added. The sample was allowed to stand for 60 min at 70°C in an oil bath for silylation. It was then cooled to room temperature, mixed with 50 µl retention time standard 2 and 1 µl was analyzed by gas chromatography.

3.2.2 Gas chromatography

Gas chromatography (GC) was performed on a Focus GC (Thermo, Austin, TX) equipped with a flame ionization detector (FID).

GC conditions:

Injection volume:	1 µL
Column:	DB1, J & W Scientific, Folsom, CA 60 m x 0.32 mm i.d. fused silica capillary coated with a 0.25 µm film of polydimethylsiloxane
Injector type:	Split / Splitless
Split flow:	25 mL/min
Carrier gas:	Hydrogen
Flow modus:	1.8 mL, constant flow
Injector temperature:	250 °C
Detector temperature:	320 °C
Temperature program:	100 °C to 320 °C at 4 °C/min, 25 min hold

GC-FID data were acquired using the commercially available software Chrom-Card 2.3 (Thermo Electron). The sampling rate was set to 600 data points/min. Automated peak integration within the retention time range between 5 min and 50 min was performed using a peak width of 5 sec and a peak threshold of 5. For integration of peaks eluting at retention times higher than 50 min, peak threshold was set to 9 and peak width was adjusted to 15 sec. Peaks exhibiting an area lower than 3000 µV*min were not taken into account.

3.2.3 Gas chromatography – mass spectrometry

Gas chromatography – mass spectrometry (GC-MS) analysis was performed on a Finnigan TraceGC ultra coupled to a quadrupole mass selective detector Finnigan Trace DSQ (Thermo, Austin, TX).

GC conditions:

Injection volume:	1 µL
Column:	FactorFOUR VF-1ms, Varian

	60 m x 0.32 mm i.d. fused silica capillary coated with a 0.25 µm film of polydimethylsiloxane
Injector type:	Split / Splitless
Split flow:	15 mL/min
Carrier gas:	Helium
Flow modus:	1.0 mL, constant flow
Injector temperature:	280 °C
Detector temperature:	320 °C
Temperature program:	100 °C to 320 °C at 4 °C/min, 10 min hold
MS conditions:	
Interface temperature:	320 °C
Ionization method:	EI+, 70 eV
Source temperature:	250 °C
Scan range:	40 – 700 Da (full scan mode)
Scan interval:	0.4 s

GC-MS data were acquired using the commercially available software Xcalibur 1.4 SR1 (Thermo, Austin, TX).

3.2.4 Identification of metabolites

Identification of mung bean constituents was achieved by comparison of retention times and mass spectra with those of silylated and methylated reference compounds, by comparison with mass spectral data and retention time indices of the GOLM metabolome data base (Kopka et al., 2005) and by comparison of mass spectra with entries of mass spectra library NIST02 and from literature (Xu and Godber 1999, Kamal-Eldin et al. 1992).

3.2.5 Recovery rates

Recoveries were determined by spiking the starting material (0h) and mung beans germinated for 75h with known amounts of reference standards. The samples were analyzed as described above. In addition, samples of the same mung bean flour were analyzed without spiking. There are 3 sets of aliquot. The first aliquot of the extract was spiked with the standard compounds at the beginning of the analytical procedure. The second aliquot was spiked at the end of the work up prior to GC/MS investigation. The third aliquot of the extract was analyzed to calculate peak heights of the standard compounds naturally observed in the unspiked flour. Peak heights of the first extract aliquot were compared to peak heights of the second sample, taking the peak heights of extract aliquot three into account. Recoveries were calculated according equation 1.

$$W = \frac{H_{P1} - H_{P3}}{H_{P2} - H_{P3}} * 100\%$$

Equation 1: Calculation of recovery rates: W recovery rate, H_{P1} peak height in the aliquot of the extract spiked in the beginning of the analytical procedure, H_{P2} peak height in the aliquot of the extract spiked prior to GC/MS investigation, H_{P3} peak height in the un-spiked aliquot of the extract.

3.2.6 Preparation of standard compounds for recovery rate

Fraction I: 20 µL of *n*-hexane solution containing 9.5 mg/ml tripalmitin, 5.0 mg/ml trimargarin, 5.0 mg/ml tristearin, 1.5 mg/ml triarachin and 0.85 mg/ml squalene was added to the flour at the start of analysis. 20 µl of *n*-hexane solution containing 9.5 mg/mL methyl palmitate, 5.0 mg/ml methyl margarate, 5.0 mg/ml methyl stearate, 1.5 mg/ml methyl arachidonate and 0.85 mg/ml squalene was added to the sample prior to GC/FID analysis.

Fraction II: 20 µL of dichloromethane solution containing 0.85 mg/ml palmitic acid, 0.85 mg/ml stearic acid, 0.90 mg/ml phytol, 1.5 mg/ml cholesterol, 1.0 mg/ml stigmasterol and 0.85 mg/ml α-tocopherol was added to the mung bean flour and to fraction II prior to silylation, respectively.

Fraction III: 20 mg sucrose and 100 µl of hexane solution containing 1.0 mg/ml fructose, 1.0 mg/ml glucose, 1.0 mg/ml raffinose, 1.5 mg/ml stachyose, 1.0 mg/ml

galactitol and 1.0 mg/ml *myo*-inositol (standard stock solution for fraction III) were added to the flour at the start of analysis. At the end of analysis, 75 µL of a pyridine/TMSIM (50+25, v+v) solution of 6 mg sucrose was added to 150 µl of upper phase of fraction III.

Fraction IV: 50 µL of an aqueous solution containing 0.8 mg/ml glycine, 0.5 mg/ml aspartic acid, 0.8 mg/ml glutamine, 0.1 mg/ml lactic acid, 0.8 mg/ml fumaric acid and 0.8 mg/ml malic acid was added to 2 ml of polar extract and to fraction IV prior to silylation, respectively.

3.2.7 Repeatability

Repeatability was determined by triplicate analysis of the mung bean samples. Relative standard deviations (RSD) of peak heights normalized to the internal standard of the respective fraction were calculated.

3.2.8 Statistical assessment

Mung bean samples were analyzed in triplicate. Repeatability was determined by triplicate analysis of a mung bean sample. Relative standard deviations (RSD) of peak heights were calculated using normalized peak heights to the internal standard of the respective fraction. GC-FID data were acquired and integrated using Chrom-Card 2.3 (Thermo Electron, Italy). Peak heights and retention times were exported to Chrom*pare* 1.1 (http://www.chrompare.com) (Frenzel et al., 2003) for standardization for peak heights on the basis of internal standards added prior to the fractionation process and for correction of peak retention time shifts on the basis of retention time standards. Principal component analysis (PCA) was performed using XLSTAT 7.5.2 (Addinsoft, France).

4 RESULTS AND DISCUSSION

4.1 METABOLITE PROFILING OF INTACT MUNG BEANS

Metabolite profiling represents the analysis of selected compounds from the same chemical compound classes or compounds linked by known metabolic relationships. It aims at the detection, identification and quantification of a broad spectrum of compounds in a single sample. The metabolite profiling procedure developed for the analysis mung beans can be subdivided into the following principle steps:

- Sample preparation
- Extraction and fractionation of metabolites
- GC analysis of metabolites
- Data processing and statistical analysis.

Prior to extraction of the metabolites, it has to be made sure that all metabolic processes in the mung beans are stopped. Freezing the material in liquid nitrogen and subsequent freeze-drying was applied within the metabolite profiling sample preparation. For the extraction of metabolites, pure organic solvents or mixtures of solvents are added to the milled plant samples. Polar metabolites are usually extracted with methanol, ethanol and water, whereas chloroform, chloroform / methanol mixtures or dichloromethane are used for the extraction of lipophilic compounds from the biological matrix. The extraction and fractionation scheme applied for the metabolite profiling of mung beans and mung bean sprouts is shown in Figure 6. Originally, it was developed for the metabolite profiling of rice (Frenzel et al., 2002) and has since been adopted for unbiased analyses of maize (Röhlig et al., 2009), barley (Frank et al., 2011) and soybean (Frank et al., 2009). The approach, based on consecutive extraction of lipids and polar compounds, is comparable to other profiling strategies with respect to the extraction of metabolites with solvents differing in polarity. However, additional transesterification-solid phase extraction (lipids) and selective hydrolysis of silylated derivatives (polar compounds) were applied to separate major from minor constituents. This procedure results in four fractions containing fatty acid methyl esters and hydrocarbons (fraction I), free fatty acids, alcohols and sterols (fraction II), sugars and sugar alcohols (fraction III), acids, amino acids and amines (fraction IV).

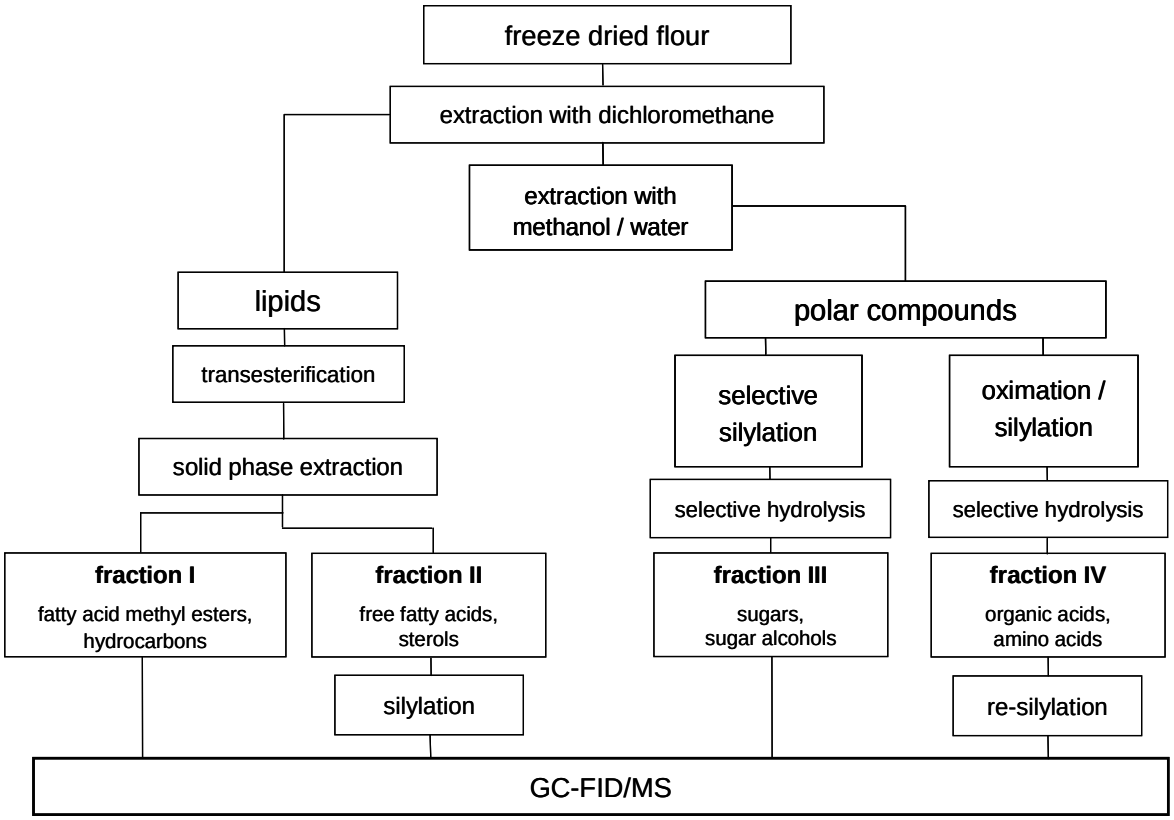


Figure 6: Extraction and fractionation of freeze-dried mung bean flour.

4.1.1 Detection and Identification of Metabolites

Freeze-dried mung bean flour was subjected to the extraction and fractionation sequence described above. The obtained non-polar and polar fractions were subjected to GC-FID and GC-MS analyses. GC-FID chromatograms of the four fractions obtained from mung beans are shown in Figure 7. The applied GC analysis resulted in the detection of a total of 456 distinct analytes of which 146 compounds could be identified based on the comparison of mass spectral data and retention times to those of reference compounds or to literature data. The compounds identified in the non-polar and polar fractions are presented in Tables 1 and 2, respectively. The number of identified compounds in mung beans is in the same order of magnitude as for comparable metabolite profiling studies on rice (Shu et al., 2008), barley (Frank et al., 2011) and maize (Röhlig et al., 2009). For wheat, metabolite profiling allowed the detection of 250 distinct peaks and the identification of 52 compounds in the polar extract (Zörb et al., 2006).

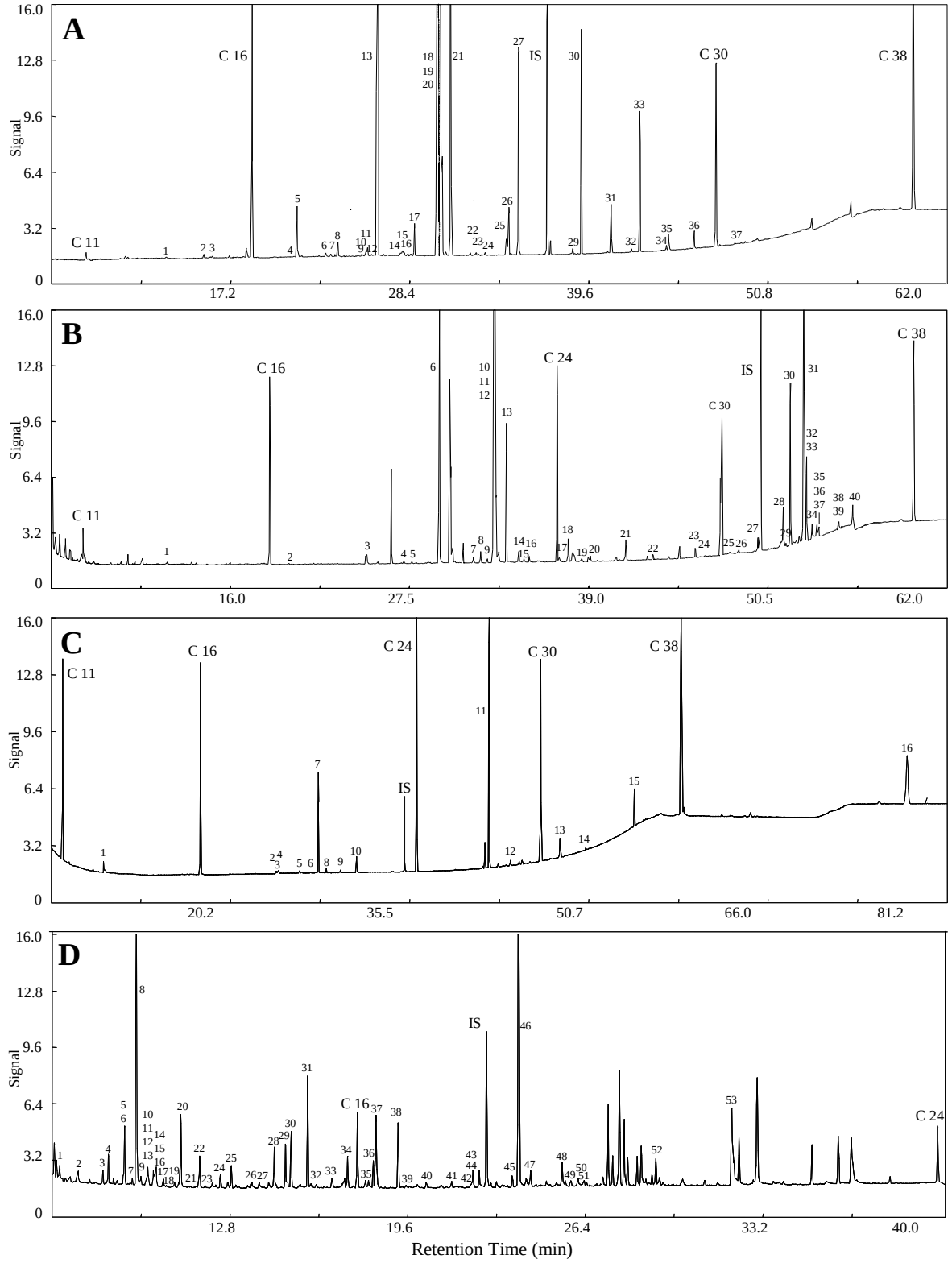


Figure 7: GC-FID chromatograms of fraction I (A), fraction II (B), fraction III (C) and fraction IV (D) obtained from mung beans. I.S.: internal standards tetradecane (A), 5α-cholestane-3ß-ol (B), phenyl-ß-D-glucopyranoside (C), *p*-chloro-L-phenylalanine (D); C16, C24, C30, C38: retention time standards; identification of peaks is given in Tables 1 and 2.

Table 1: Compounds identified in fraction I (major lipids) and TMS derivatives of compounds identified in fraction II (minor lipids)

no.	compound	ident[a]
saturated FAME[b]		
3	12:0	A
5	14:0	A
8	15:0	A
13	16:0	A
17	17:0	A
21	18:0	A
24	19:0	A
27	20:0	A
28	21:0	A
30	22:0	A
31	23:0	A
33	24:0	A
34	25:0	C
36	26:0	A
37	28:0	A
free fatty acids[c]		
1	9:0	A
2	12:0	A
3	14:0	A
4	15:0	A
6	16:0	A
7	17:0	C
10	18:3 (9Z, 12Z, 15Z)	A
11	18:2 (9Z, 12Z)	A
12	18:1 (9Z)	A
13	18:0	A
15	19:0	A
17	20:1 (11Z)	A
18	20:0	A
21	22:0	A
22	23:0	A
23	24:0	A

no.	compound	ident[a]
unsaturated FAME[b]		
6	15:1 (10Z)	A
10	16:1 (9Z)	A
11	16:1 (9E)	A
15	17:1 (9Z)	A
20	18:1 (9Z)	A
22	19:1 (10Z)	B,C
26	20:1 (11Z)	A
29	22:1 (11Z)	A
32	24:1 (11Z)	A
9	16:2	B,C
14	17:2	C
19	18:2 (9Z, 12Z)	A
25	20:2 (11Z, 14Z)	A
18	18:3 (9Z, 12Z, 15Z)	A
fatty alcohols[c]		
5	16:0	A
8	18:0	A
9	phytol	A
16	20:0	A
20	22:0	A
38	32:0	D
hydroxy FAME[b,c]		
14	12-OH 18:1 (9Z)	A
19	9,12-OH 18:0	E
tocopherols[c]		
24	δ-tocopherol	A
25	γ-tocopherol	B,C
26	α-tocopherol	A

no.	compound	ident[a]
hydrocarbons		
1	C14	A
2	C15	A
4	C17	A
7	C18	A
12	C19	A
16	C20	A
23	C22	A
35	squalene	A
sterols/stanols[c]		
27	cholesterol	A
28	campesterol	A
29	campestanol	A
30	stigmasterol	A
31	β-sitosterol	A
32	sitostanol	A
33	Δ5-avenasterol	A
34	gramisterol	F
35	Δ7-stigmastenol	F
36	cycloartenol	A
37	Δ7-avenasterol	D,F
39	24-methylene-cycloartanol	A
40	citrostadienol	F

[a] Identification according to
A mass spectral data and retention times of reference compounds
B mass spectral data and retention index of Golm Metabolome Database (Kopka *et al.*, 2005)
C mass spectral data of NIST02 mass spectral library
D mass spectral data
E Xu and Godber, 1999
F Kamal-Eldin et al., 1992
[b] fatty acid methyl esters
[c] TMS derivatives of respective compound

Table 2: TMS derivatives of compounds identified in fraction III (sugars and sugar alcohols) and fraction IV (acids, amino acids and amines)

no.	compound[a]	ident[b]	no.	compound[a]	ident[b]
sugars and sugar alcohols			*amino acids and amines*		
1	glycerol	A	3	alanine	A
2,3,4	fructose	A	4,17	glycine	A
5,8	galactose	A	5	valine	A
6,9	glucose	A	6	norvaline	A
7	galactitol	A	9	leucine	A
10	*myo*-inositol	A	10	ethanolamine	A
11	sucrose	A	12	alloisoleucine	A
12	trehalose	A	14	isoleucine	A
13	galactopinitol B	A	15	proline	A
14	galactinol	A	22	serine	A
15	raffinose	A	24	threonine	A
16	stachyose	A	26	methyl-L-cysteine	A
			27	β-alanine	A
acids			30	pyroglutamic acid	A
1	lactic acid	A	31	aspartic acid	A
2	propanoic acid	A	32	γ-aminobutyric acid	A
7	4-hydroxybutyric acid	A	33	5-hydroxynorvaline	C
8	phosphoric acid	A	34	threonic acid	A
11	maleic acid	A	36	glutamic acid	A
13	4-aminobutyric acid	A	37	phenylalanine	A
16	succinic acid	A	39	asparagine	A
19	glyceric acid	A	42	putrescine	A
20	fumaric acid	A	43	glutamine	A
21	pyrrole-2-carboxylic acid (2TMS)	A	45	arginine	A
23	glutaric acid	A	48	histidine	A
25	benzoic acid	A	50	lysine	A
28	amino malonic acid	C	51	tyrosine	A
29	malic acid	A	53	tryptophan	A
35	β-hydroxy-β-methyl-glutaric acid	A			
38	tartaric acid	C	*others*		
40	α-aminoadipic acid	A	18	2,4-hydroxy-pyrimidine	A
41	cis-aconitic acid	B,C	47	adenine	A
44	3-glycerophosphoric acid	A			
46	citric acid	A			
49	p-cumaric acid	A			
52	glucaric acid	C			

[a] Metabolites identified as persilylated derivates, number of trimethylsilyl (TMS) groups in parentheses

[b] Identification according to

A mass spectral data and retention times of reference compound
B mass spectral data and retention index of Golm Metabolome Database (Kopka *et al.*, 2005)
C NIST02 mass spectral library

4.1.2 Recovery and repeatability

For the determination of recovery rates in intact mung bean seeds, 300 mg of freeze-dried flour was used for the metabolite profiling procedure. The results are presented in Table 3.

Table 3: Mean recoveries (*n*=3) and repeatability rates expressed as relative standard deviation (RSD) of three-fold work-ups calculated for selected compounds from intact mung bean seeds.

compound	recovery [%]	RSD [%]	compound	recovery [%]	RSD [%]
fraction I			*fraction III*	102 ± 9	5
C16:0[a]	98 ± 4	5	fructose	107 ± 1	11
C18:0[a]	107 ± 5	6	glucose	83 ± 10	9
C20:0[a]	100 ± 4	3	raffinose	78 ± 11	4
squalene	101 ± 6	2	stachyose	80 ± 11	6
			myo-inositol		
fraction II			*fraction IV*	118 ± 10	6
C16:0[b]	104 ± 6	6	glycine	97 ± 8	2
C18:0[b]	102 ± 3	3	aspartic acid	93 ± 12	10
phytol	96 ± 1	9	glutamine	90 ± 12	8
stigmasterol	93 ± 8	3	lactic acid	112 ± 14	3
α-tocopherol	107 ± 9	12	fumaric acid	92 ± 7	7
			malic acid		

[a] fatty acid methyl ester
[b] free fatty acid

The mean recoveries for selected representatives from different chemical classes in the non-germinated material ranged from 78 to 118% (Table 3). They are comparable to the recovery rates reported for rice (Frenzel et al., 2002) and maize (Röhlig et al., 2009) using the same methodological approach. The intra-laboratory repeatability, expressed as relative standard deviations of peak heights determined by triplicate analysis for selected representatives from the different chemical classes in intact seeds is also shown in Table 3. The mean relative standard deviation for the single fractions is 6% which is acceptable for the metabolite profiling approach.

4.2 METABOLITE PROFILING OF SPROUTING MUNG BEANS

4.2.1 Introduction

During the past years, metabolomics has evolved into an important analytical approach for the assessment of genotypic and phenotypic diversity in plants (Davies, 2007; Saito and Matsuda, 2010). It aims at the simultaneous detection, identification and quantification of a broad spectrum of compounds from different chemical classes to provide a comprehensive picture of the metabolic profiles in complex biological systems. Various techniques including GC/MS, LC/MS and NMR have been employed for metabolomics-based investigations of foods (Cevallos-Cevallos et al., 2009). Among the different metabolomics approaches, GC-based metabolite profiling represents a relatively inexpensive, robust and mature technology (Wishart, 2008). In the field of plant metabolomics, the influence of different breeding systems (Frank et al., 2009), farming practices (Röhlig and Engel, 2010) and environmental factors (Röhlig et al., 2009) on crop metabolic profiles was investigated by comparative GC-based metabolite profiling demonstrating its potential to provide valuable information regarding crop composition and metabolic interactions. Understanding these metabolic interactions is a major goal in systems biology (Saito and Matsuda, 2010) and is suggested to assist in the elucidation of plant functional genomics (Fiehn et al., 2000b). Metabolite profiling is also considered to provide valuable data for breeding-driven metabolic engineering of nutritionally important metabolites in crops (Hall et al., 2008) and thus as a tool to improve their agronomic and nutritional characteristics (Dixon et al., 2006; Harrigan, et al., 2007).

In addition to the analysis of physiologically mature crops, metabolite profiling of plant developmental systems has gained increasing attention. Metabolite profiling has been employed to follow naturally occurring germination processes in rice (Shu et al., 2008) and in potatoes (Shepherd et al., 2010). In addition, malting of barley, as an example of processing-related germination has been followed via metabolite profiling (Frank et al., 2011).

To date, only few GC-based metabolite profiling studies on legumes have been carried out (Thompson et al., 2009). For example, studies on *Lotus japonicus* (Desbrosses et al., 2005) and soybeans (Frank et al., 2009) have been conducted. A

metabolomics-based investigation of mung bean seeds and the sprouting process has not yet been described. Therefore, the aim of this study was (i) to cover a broad spectrum of low molecular weight constituents in mung bean seeds by applying a GC-based metabolite profiling method, (ii) to test the employed approach regarding its suitability to reflect the germination process by a time-dependent clustering based on multivariate analysis, and (iii) to quantify major constituents and to follow their dynamic changes during sprouting.

4.2.2 Seed sprouting

The mung bean seeds were cleaned and rinsed with tap water before they were soaked for 12 at 35°C. The soaked seeds were placed between moist filter paper in petri dishes and incubated at 35°C. Samples were taken after 0h (before soaking), 24h, 36h, 48h, 60h and 75h. The developmental changes observed in the course of the sprouting are shown in Figure 8. The development of the dicotyledonous plants is accompanied by morphological changes involving the initial enlargement of the embryo and the development of cotyledon, roots, hypocotyl, shoots and leaves. The lengths of the mung bean sprouts consistently increased in the course of the germination process from around 1 cm after 24h of incubation (Figure 8B) to 8-9 cm after 75 hours (Figure 8F).

Figure 8: Intact mung beans (A) and seeds germinated for 24h (B), 36h (C), 48h (D), 60h (E) and 75h (F).

4.2.3 Analysis of sprouts

4.2.3.1 Methodology

For intact mung bean seeds, an amount of 300 mg of freeze-dried flour was used for the metabolite profiling extraction and fractionation procedure. However, the work-up of 300 mg of mung bean sprouts (after 75 h of incubation) resulted in poor recovery rates, in particular for the polar compounds (Table 4). The chromatograms of fraction IV shown in Figure 9 revealed significant increases in the levels of low molecular weight polar compounds during the germination process. To avoid potential overloading effects during extraction and fractionation, for beans germinated for 36 h and longer only 100 mg of freeze-dried flour were subjected to the work-up procedure. The resulting recovery rates were comparable to those obtained for the respective fractions from the intact mung beans (Table 4). The intra-laboratory repeatability, expressed as relative standard deviations of peak heights determined by triplicate analysis for selected representatives from the different chemical classes in intact seeds and mung bean sprouts, is shown in Table 4.

4.2.3.2 Multivariate data assessment

The consolidated metabolite profiling data were assessed via multivariate and univariate analyses.

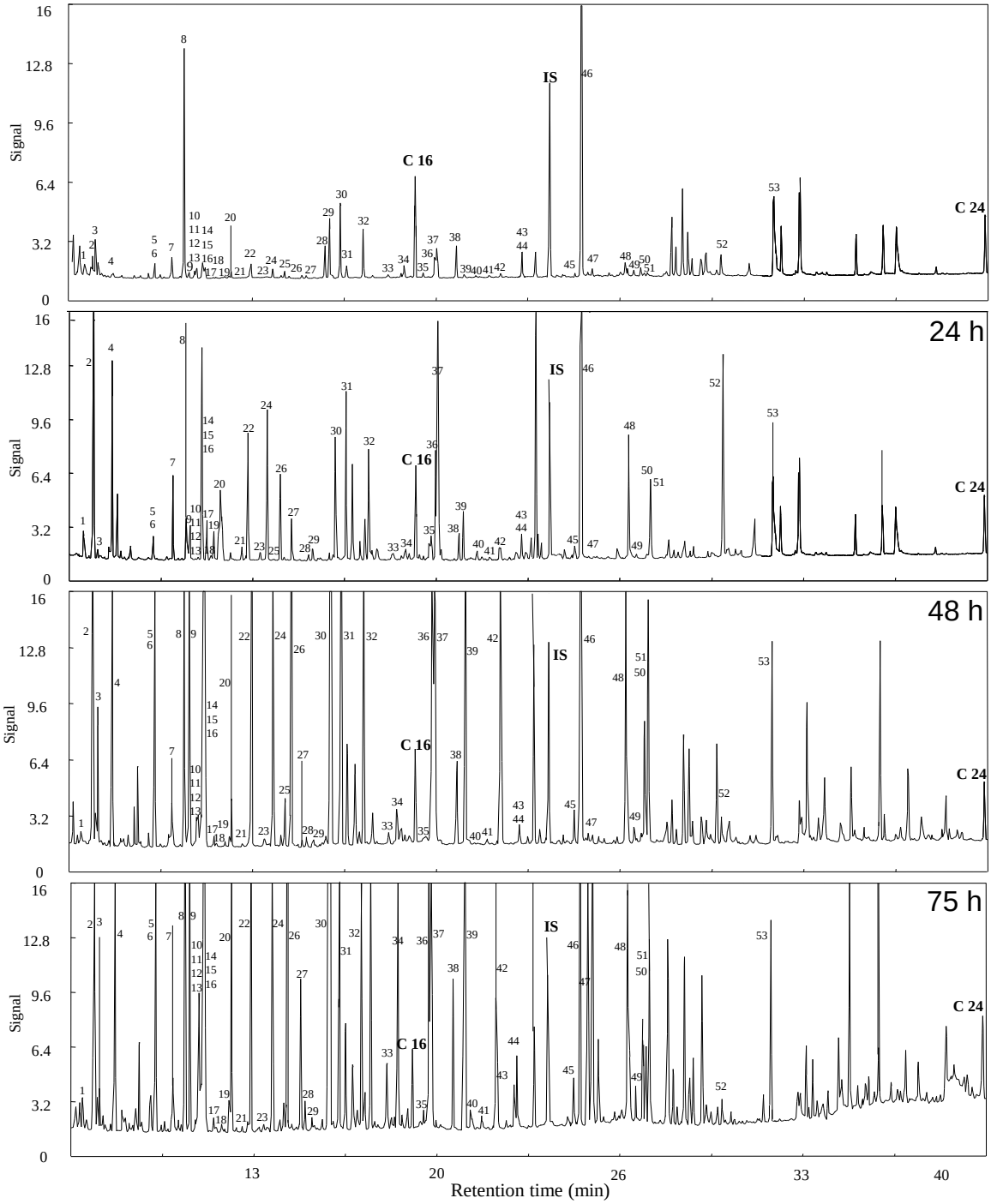


Figure 9: GC/FID chromatograms of fractions IV obtained for the mung beans during the germination after 0h (A), 24h (B), 48h (C) and 75h (D).

The data (peak heights and corresponding retention times) were standardized by means of Chrom*pare*, a software tool developed for comparative analysis of metabolite profiling data (Frenzel et al., 2003). Multivariate analysis was conducted by means of principal component analysis (PCA) for each single fraction I to IV and additionally for the combined fractions.

Table 4: Mean recoveries (*n*=3) and repeatability rates expressed as relative standard deviation (RSD) of three-fold work-ups calculated for selected compounds from mung bean sprouts (after 75 h of incubation).

Compound	Recovery [%]			RSD [%]		
	0h	75h		0h	75h	
	300mg[a]	300mg[a]	100mg[a]	300mg[a]	300mg[a]	100mg[a]
fraction I						
C16:0[b]	98 ± 4	104 ± 10	107 ± 11	5	4	1
C18:0[b]	107 ± 5	104 ± 12	99 ± 4	6	3	7
C20:0[b]	100 ± 4	104 ± 1	109 ± 12	3	2	4
squalene	101 ± 6	103 ± 3	103 ± 6	2	1	5
fraction II						
C16:0[c]	104 ± 6	85 ± 11	88 ± 3	6	12	10
C18:0[c]	102 ± 3	97 ± 8	94 ± 2	3	19	9
phytol	96 ± 1	108 ± 8	104 ± 6	9	6	7
stigmasterol	93 ± 8	119 ± 11	86 ± 9	3	9	4
α-tocopherol	107 ± 9	106 ± 10	98 ± 8	12	8	10
fraction III						
fructose	102 ± 9	32 ± 14	95 ± 8	5	9	7
glucose	107 ± 1	61 ± 6	81 ± 12	11	28	7
raffinose	83 ± 10	65 ± 7	93 ± 1	9	17	8
stachyose	78 ± 11	63 ± 7	95 ± 8	4	15	4
myo-inositol	80 ± 11	71 ± 4	95 ± 6	6	5	9
fraction IV						
glycine	118 ± 10	83 ± 6	94 ± 9	6	16	5
aspartic acid	97 ± 8	63 ± 13	103 ± 4	2	9	3
glutamine	93 ± 12	82 ± 11	102 ± 5	10	35	8
lactic acid	90 ± 12	70 ± 1	116 ± 13	8	19	10
fumaric acid	112 ± 14	98 ± 7	84 ± 6	3	13	4
malic acid	92 ± 7	38 ± 7	85 ± 5	7	10	7

[a] fatty acid methyl ester
[b] free fatty acid

The scores plots obtained for the data on mung beans at different stages of the sprouting process are shown in Figure 10. The metabolic changes are reflected by time-dependent shifts of the scores for the first two principal components PC1 and PC2. For the combined non-polar and polar fractions (I-IV), a total variance of 60% was covered by PC1 and PC2 (Figure 10A). Comparable coverage rates were observed for the single fractions (Figure 10B-E). Similar coverage rates have also been reported for rice (Shu et al., 2008) and potato (Davies, 2007) during germination.

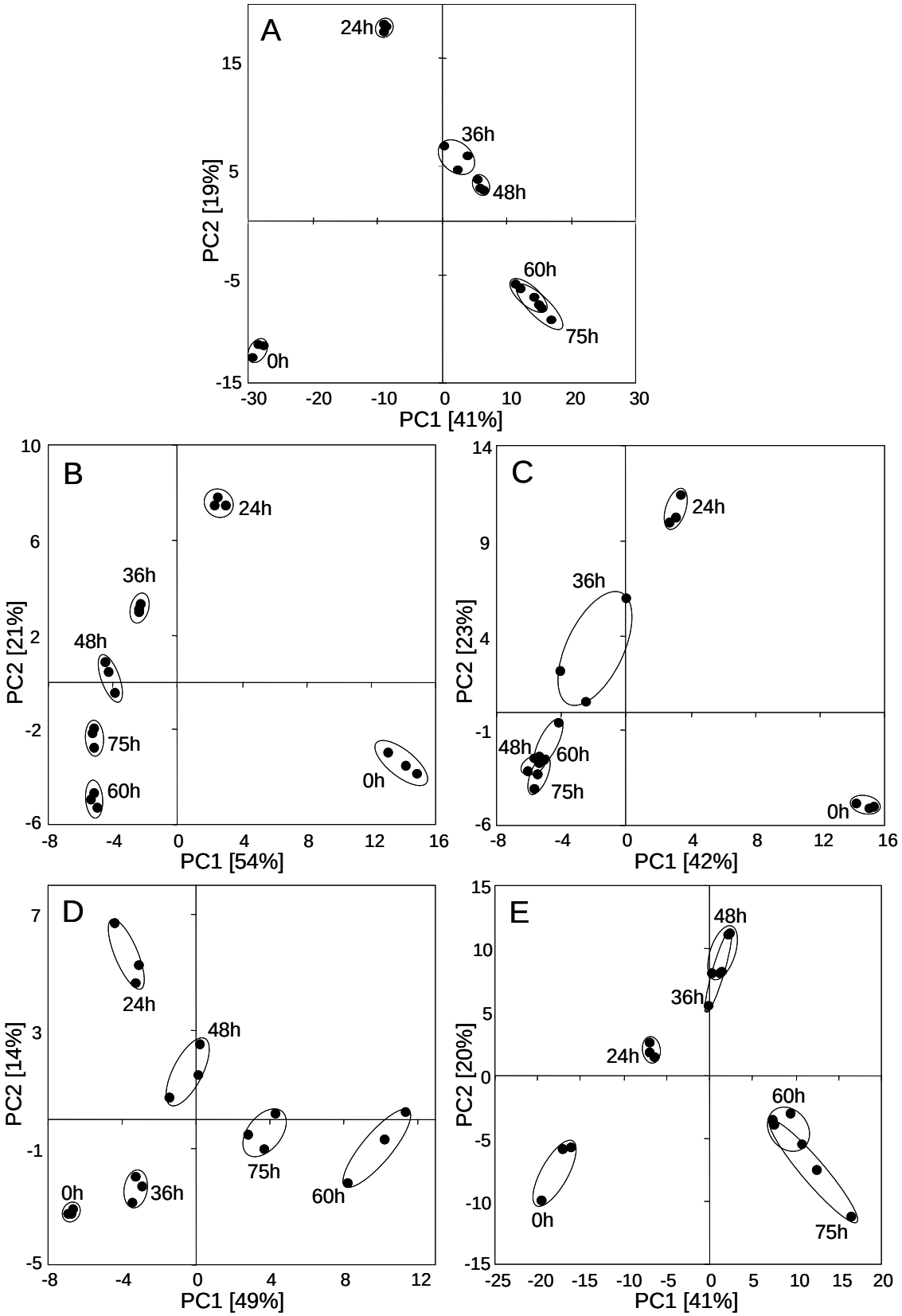


Figure 10: Principal component analysis (PCA) of standardized GC/FID metabolite profiling data of the combined fractions I-IV (A) and the single fractions I (B), II (C), III (D) and IV (E) in the course of germination (0, 24, 36, 48, 60, 75h) of mung beans.

A nearly identical score pattern for the combined fractions I-IV was obtained in another independently performed sprouting process with mung beans from the same batch (Figure 11). This demonstrates the robustness of the applied methodology.

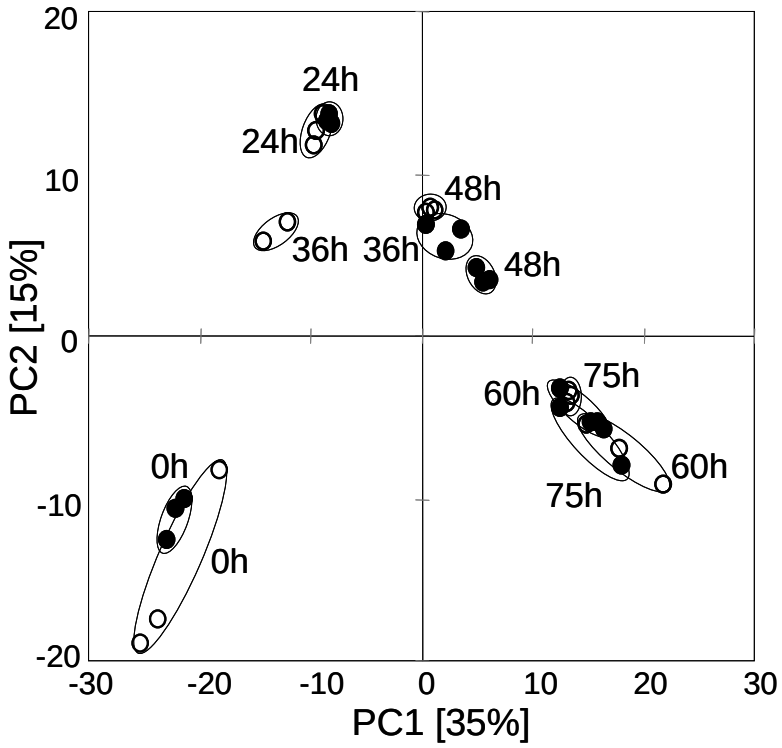


Figure 11: PCA of standardized GC/FID metabolite profiling data of the combined fractions I-IV (A) of two separately performed germination processes (time lag of one month) with the same batch of mung beans. Black and white circles indicate the two germination procedures.

In order to assess major contributors to the time-dependent separation of the sprouting mung bean samples, the PCA loading scores for the combined and single fractions were analyzed taking into account the data of all 456 detected peaks (Figure 12A-E). Polar metabolites were found to be more pronounced contributors to the separation along the first principal component than lipids (Figure 12A).

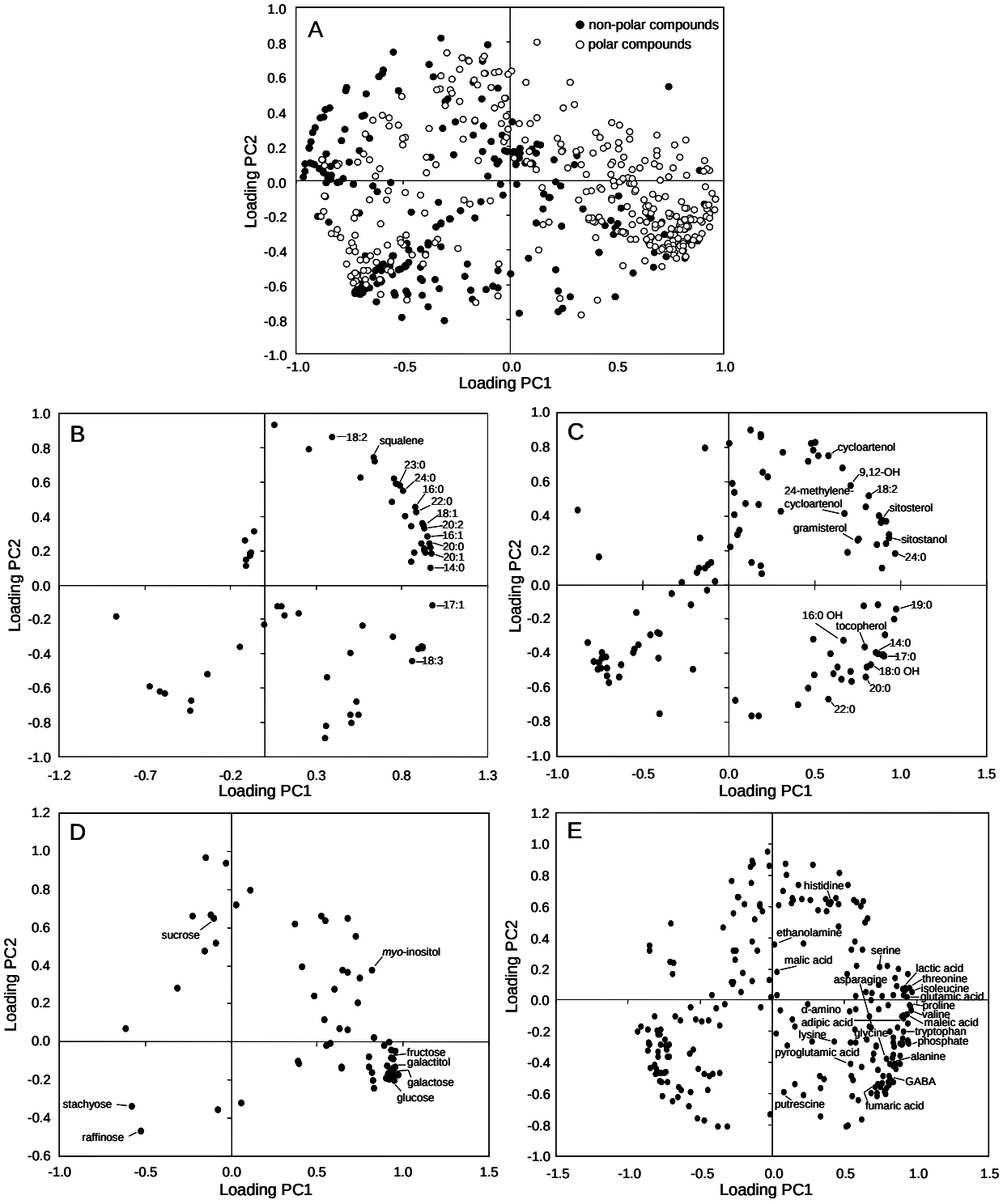


Figure 12: Loading plots of standardized GC/FID metabolite profiling data from the combined non-polar and polar fractions I-IV (A) and from the single fractions I (B), II (C), III (D) and IV (E).

4.2.3.3 Univariate data assessment

The dynamic changes of single metabolites during the sprouting process are illustrated in Figure 13 by means of a heatmap. In accordance with the results obtained by the PCA loadings (Figure 12), lipids were found to be predominantly decreased in the sprouts, whereas polar constituents exhibited mainly elevated levels compared to the intact mung bean seeds. For representatives of the polar and non-polar fractions quantitative changes in the course of the sprouting process are shown in Figure 14.

Fraction I

Fraction I mainly covers fatty acid methyl esters (FAME) that result from transesterification of the crude lipid extract and reflect the mung bean triglycerides. Within the first 24 hours of incubation, changes of most FAME levels were relatively small. However, after this initial soaking and early germination phase, the mung bean samples exhibited significantly decreased contents of fatty acid methyl esters (Figures 13A and 14A). This is an expected result as fat serves as a major energy source for the developing embryo (King and Puwastien, 1987). It is in agreement with the significant decrease (-40%) in total triglyceride content reported for mung beans after a germination period of 72 hours at 20 - 23°C (Abdel-Rahman et al., 2007). Significant reductions in FAME have also been observed in the course of the germination of rice seeds (Shu et al., 2008) and soybeans (Chandrasiri et al., 1990) and during the malting of barley (Frank et al., 2011).

Fraction II

Compared to the considerable quantitative changes in fractions I, the changes in fraction II were less pronounced. The levels of free fatty acids were the highest during the first 24 hours. After this initial phase, a significant decrease followed by a slight increase was observed (Figures 13A and 14B). The changes reported for free fatty acids during the germination of crops in the literature are not consistent. In contrast to the present study, contents of free fatty acids were found to be increased (+72%) in mung beans sprouts (Abdel-Rahmann et al., 2007) but decreased in soybean sprouts (- 68%) (McKinney et al., 1958) after a germination period of 72 hours at comparable temperatures.

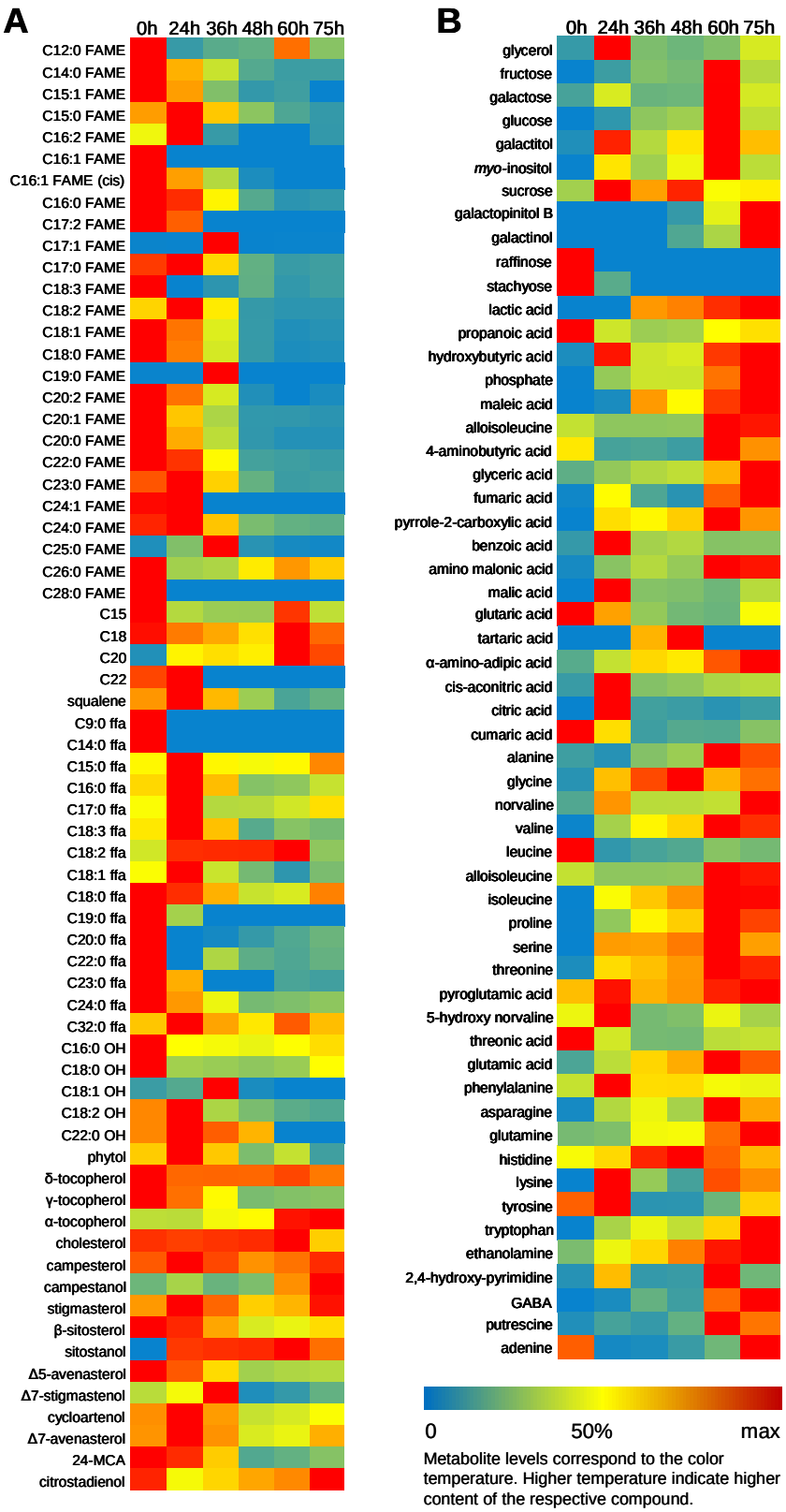


Figure 13: Heatmaps of non-polar (A) and polar (B) metabolite fractions in the course of malting. Metabolite levels correspond to the color temperature. Higher temperature indicates higher levels of the respective compound. FAME: fatty acid methyl ester, ffa: free fatty acid.

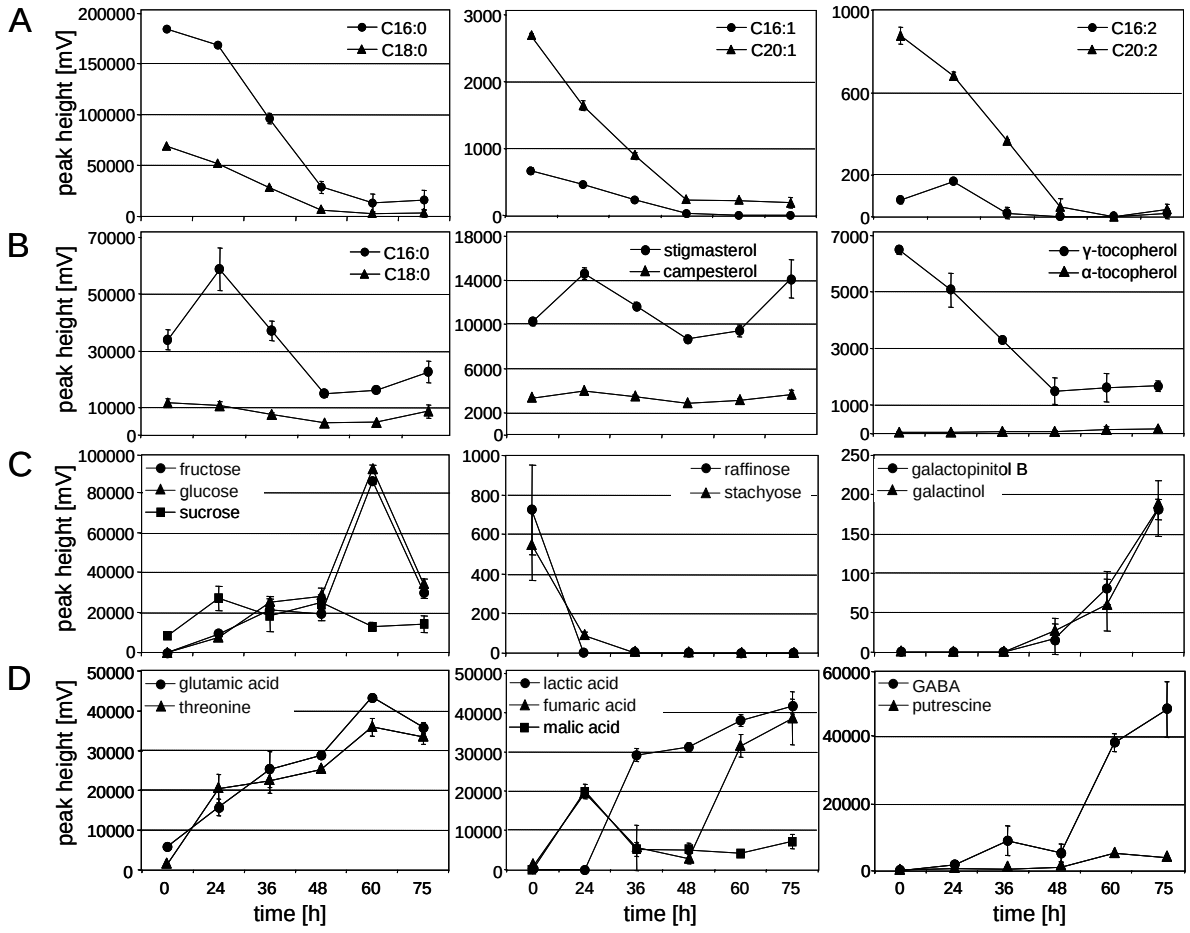


Figure 14: Standardized peak heights for selected compounds determined in fractions I (A), II (B), III (C) and IV (D) in the course of the sprouting of mung beans.

Decreased free fatty acid contents have also been reported for germinated sunflower (Munshi et al., 2007) and rice seeds (Shu et al., 2008). Regarding the dynamic changes of fatty alcohols, no consistent trend has been observed during the whole sprouting procedure.

Changes in sterol levels were relatively small. For example, the level of stigmasterol, a major sterol in mung beans, exhibited a U-shape pattern in the course of the germination (Figure 14B). A two-fold increase in total phytosterol content comprising campesterol, stigmasterol and ß-sitosterol has been determined for soybean germinated for 3 days at 25 °C (Shi et al., 2010). In another study on soybeans, only a small increase (on average +30%) has been observed within a 120 h germination period (Chandrasiri et al., 1990) indicating a significant influence on the respective raw material and incubation conditions.

In the present study, γ-tocopherol was found to be by far the major tocopherol in mung bean seeds. The ratio between α- and γ-tocopherol was 1 : 60. Mean ratios from 1 : 26 and 1 : 139 have been reported for hull-free mung been seeds (Anwar et al., 2007) and the oil fraction of mung beans, respectively (Zia-Ul-Haq et al., 2008). Although a slight increase of α-tocopherol was observed during sprouting, its total level remained low. A significant reduction by approximately -70% was observed for γ-tocopherol in the mung bean sprouts (75h) compared to the intact seeds (Figure 14B). For soybean, a three-day germination period at 25 °C resulted in a slightly increased level of γ-tocopherol (~ +35%) (Shi et al., 2010). However, after a total germination time of 7 days, the level was reduced by approximately -45% compared to the ungerminated seeds. In addition, a significant reduction in γ-tocopherol has been determined in canola oil during seed germination at 20 °C (Zhang et al., 2007). This study also demonstrated a considerable influence of illumination on the tocopherol content during germination.

Fraction III

As shown in the PCA loadings for the combined fractions (Figure 12A), the contribution of the polar metabolites to the overall changes in the metabolic profiles of mung bean seeds during sprouting is more pronounced than that of the non-polar constituents. Until an incubation time of 60 h, the levels of the monosaccharides fructose and glucose increased drastically in the germinating material. However, a significant reduction for both sugars was determined for the final germination stage from 60 to 75 h. A similar effect was observed for the second independently performed sprouting process. The concentration of the disaccharide sucrose increased within the first 24 h, but rapidly declined after the initial germination phase. The tri- and tetrasaccharides raffinose and stachyose were drastically reduced (Figure 14C). This degradation is in good agreement with previous studies on germinated mung beans (Åman, 1979; El-Adawy et al., 2003; Mubarak 2005). The decline of sucrose contents in the latter stage of sprouting might be due to the lack of raffinose oligosaccharides resulting in the hydrolysis of sucrose for the energy supply. A decrease in the levels of raffinose oligosaccharides is a desired trait from a nutritional point of view as such constituents cannot be hydrolyzed in the intestine of humans due to the absence of the enzyme α-galactosidase (Horbowicz and Obendorf, 1994). Several galactosyl cyclitols have been described to be present in a

range of legumes (Horbowicz and Obendorf, 1994). The level of galactopinitol B in the germinated sprouts was significantly increased compared to the ungerminated seeds (Figure 14C). However, compared to the total amounts of the other sugars in fractions III, the quantitative changes in galactopinitol B were negligibly small.

Fraction IV

Compared to cereals, mung beans contain higher protein contents (Kirchoff, 2008). The proteolytic cleavage of the proteins during germination led to a significant increase in the levels of most amino acids (Figure 13B and 14D). Increased contents of free amino acids in germinated mung beans and lentils have been observed via targeted analysis (Kavas and El, 1991; Chau et al., 1997; Mubarak, 2005). Comparable GC-based metabolite profiling studies also revealed significantly higher amino acid levels in germinated rice (Shu et al., 2008) and barley (Frank et al., 2011). Phosphoric and citric acid are two of the major acids found in fraction IV. The overall levels of the acids were increased in the course of sprouting. A distinct and continuous increase in lactic acid was observed, whereas malic acid and citric acid exhibited their maximum already after 24 h of incubation (Figures 13B and 14D). Strong increases in lactic acid and malic acid contents were also found for soybeans during a 24 h of soaking at 30 °C whereas citric acid only increased during the first 6 h followed by its decrease until the end of the soaking period (Mulyowidarso et al., 1991). In this context the great influence of the microorganism flora on the acid concentrations has to be considered. Inoculation of soaking soybeans with different microorganism cultures resulted in highly variable acid concentrations (Mulyowidarso et al., 1991).

For γ-aminobutyric acid (GABA), a well known naturally occurring biogenic amine, a 26-fold increase from 24 h to the final sprouts (75 h) has been determined (Figure 14D). The precursor of GABA, glutamic acid was also significantly increased during the sprouting process. Increased levels of GABA have also been reported for germinated cereals like barley (Kihara et al., 2007) and wheat (Nagaoka, 2005). Enhanced contents of GABA in sprouts for human nutrition are of special interest because of its health-promoting impact (Ito, 2004). The biogenic amine putrescine plays important roles in plant physiology (Kusano et al., 2007) However, its increase might also reflect a certain extent of microbial contamination of the mung beans especially as the raw material was not sterilized before the sprouting process.

4.2.4 Conclusions

The data obtained demonstrate the suitability of the described metabolite profiling technique to follow metabolic changes in a complex plant matrix. Owing to inherent features of the approach, e.g. choice of extraction solvents, derivatization steps or volatilities of derivatives, the type of metabolites covered is to some extent pre-determined and not fully “unbiased”. However, the metabolites stem from a wide range of chemical classes and the number of compounds detected and identified is in an order of magnitude comparable to those reported for other GC-MS based metabolite profiling studies (Schauer et al., 2005; Tarpley et al., 2005). In addition to representatives of primary plant metabolism, nutritionally relevant metabolites are covered. They range from the lipophilic sterols to the polar amino compounds γ-aminobutyric acid and putrescine. The applied methodology is suitable to cover those metabolites shown to form a basis for metabolic investigation of sprouting mung beans by means of a GC-based approach. The metabolic changes observed during the sprouting of mung beans also confirm the potential of metabolite profiling techniques to reveal distinct metabolic switches in the developmental stages of mung bean seeds.

4.3 SPROUTING IMPACT FACTORS

4.3.1 Introduction

The metabolic phenotype of mature crop seeds is mainly defined by the genetic background (e.g. different genotypes), the breeding strategy (e.g. conventional breeding and genetic engineering), the environmental growth conditions (e.g. climatic differences during the growing seasons) and the crop management system used (e.g. conventional and organic practices). In the recent years, metabolite profiling was shown to be a suitable tool for the investigation of these impact factors on the metabolic phenotypes of mature crops (Harrigan et al., 2009; Röhlig et al., 2009; Frank et al., 2012b). In addition, metabolite profiling has been demonstrated to be a powerful tool to follow metabolic changes in plant developmental systems (Shu et al., 2008; Shepherd et al., 2010; Frank et al., 2011). The applied metabolite profiling procedure to sprouting mung beans revealed significant dynamic changes in the metabolic phenotype during germination (see Chapter 4.2).

The aim of the present chapter was to investigate the influence of genotype, growing environment, temperature, illumination and washing treatment on the metabolite profiles of sprouting mung beans. Two mung bean materials obtained from industry and from a local health-food shop, respectively, were subjected together to three sprouting processes to investigate the genetic influence on the metabolite profiles. In addition, two mung bean varieties, both grown at dry and rainy seasons in Thailand were sprouted under constant incubation conditions to analyze the impact of environment and growth condition on the metabolite profiles during the sprouting process. Further, mung beans were sprouted at different incubation temperatures. For germinating crops, the incubation temperature is known to be a major driver for the time-dependent progress. To follow the metabolic changes in the present study, mung bean materials from the same batch were sprouted at 25°C and 35°C and the metabolite profiles were assessed by means of multivariate and univariate analyses. For the investigation of the influence of illumination on the metabolite profiles of sprouting mung beans, samples were germinated under artificial light exposure and comparatively analyzed to the respectively mung beans sprouted in the dark. To investigate the impact of a washing treatment, intact mung beans were washed with

Millipore water and treated with methanol before the sprouting process. In addition, an industrial-scale mung bean sprouting including a washing step with citric acid-containing water should be compared to the same batch of mung beans sprouted without such a treatment.

4.3.2 Impact of genotype

Due to their high protein contents, mung beans and their products are important staple foods and feeds in Asian countries. With regard to the nutritional value of mung beans, breeders are interested in developing cultivars with both improved nutritional and agronomical properties. Breeding of different mung bean genotypes might also be necessary to consider different growing environments. Studies published during the last years indicate significant effects on the quality of bean seeds related to the following interactions: genotype x location, genotype x season and genotype x year (Shellie and Hosfield, 1991; Santalla et al., 1995). The data are mostly related to seed size, contents of protein, starch and soluble sugars, fat, minerals, seed hardness, water uptake by dry seeds and cooking characteristics. For the present study, two mung bean materials, one purchased at a local health-food shop and one obtained from a commercial mung bean sprout producer, were sprouted together three times under small-scale laboratory conditions. The sprouting progress is shown in Figure 15. Morphological differences during the progress were not observed.

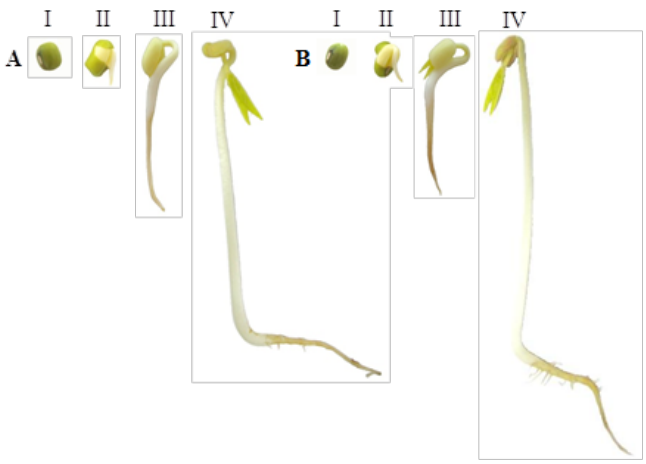


Figure 15: Mung beans obtained from a local health-food shop (AI) and a commercial sprout producer (BI) and seeds germinated for 24h (II), 48h (III) and 72h (IV).

A principal component analysis of the metabolite profiling data from the combined fractions I-IV is shown in Figure 16.

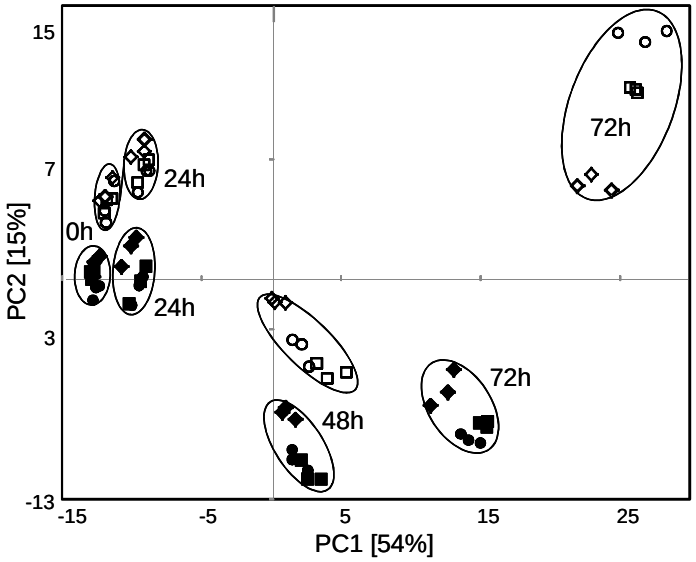


Figure 16: PCA of standardized GC metabolite profiling data of the combined fractions I-IV in the course of three independent laboratory sprouting procedures (0, 24, 48, 72h) of two mung bean materials obtained from a local store (◆,●,■) and from a commercial producer (◇,○,□).

The metabolic changes are reflected by time-dependent shifts of the scores for the first two principal components. For the combined non-polar and polar fractions, a total variance of 69% was covered by PC1 and PC2. Similar coverage rates have been reported for germinating rice and barley (Shu et al., 2008; Frank et al., 2011). PCA analysis of the metabolite profiling data revealed reproducible reflections of the three conducted sprouting processes (Figure 16). Thus, the applied methodology allowed a differentiation of the two mung bean materials in the course of germination. The material-dependent clustering observed in Figure 16 was shown to be mainly caused by the two non-polar fractions I and II containing major and minor lipids (Figure 17). Especially at the final stage of sprouting (72h), the two mung bean materials were clearly separated within fractions I and II along both principal components (Figures 17A and B), whereas the patterns seen for the polar fractions III and IV revealed less pronounced differences between the sprouting samples (Figures 17C and D).

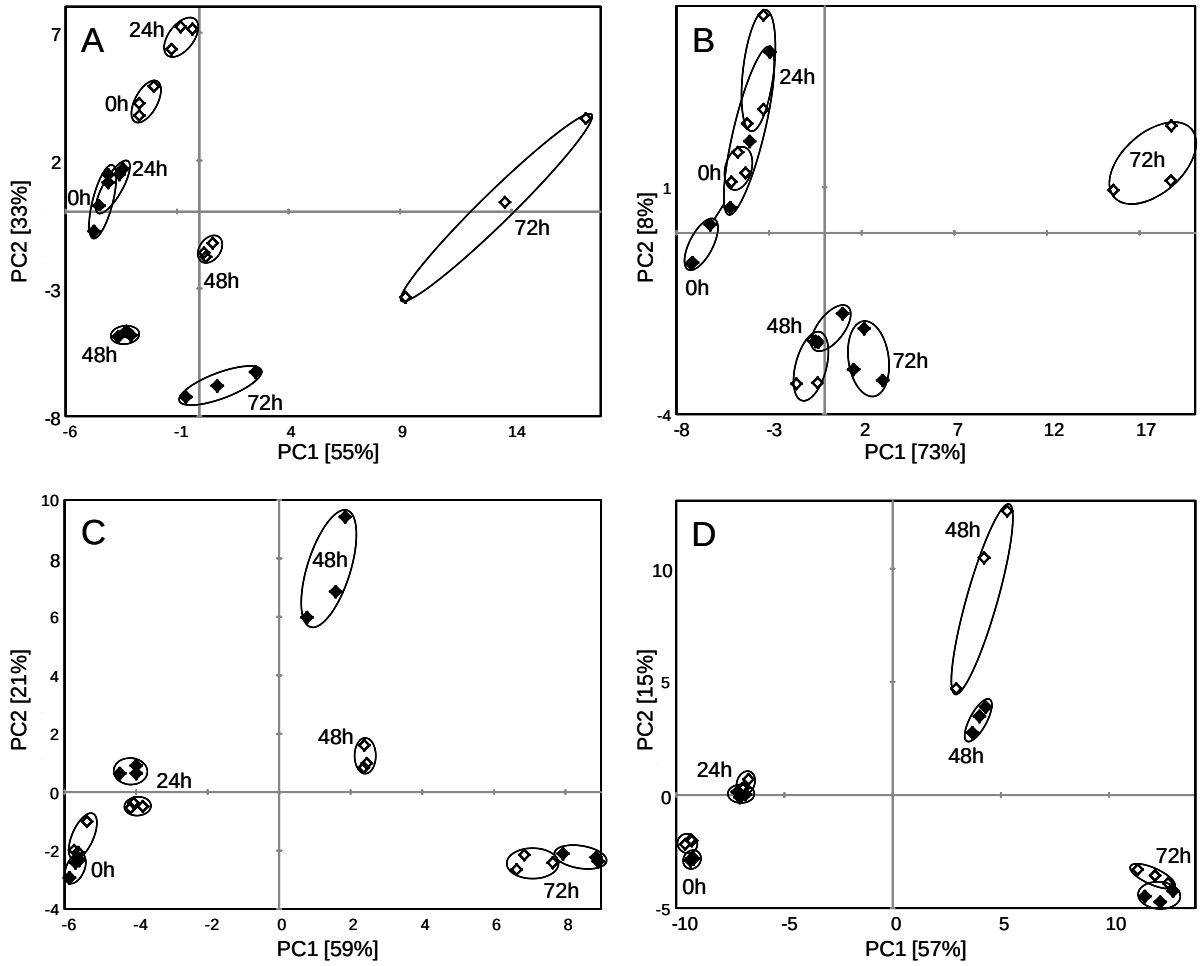


Figure 17: PCA of standardized GC metabolite profiling data of fractions I (A), II (B), III (C) and IV (D) in the course of a single laboratory sprouting process (0, 24, 48, 72h) of mung beans obtained from a local store (◆) and from commercial producer (◇).

4.3.3 Impact of environment

In this experiment, two mung bean cultivars (Chai Nat 72 and Kam Phang Saen 2) grown at rainy and dry seasons, respectively, by the Chai Nat Field Crops Research Center, Chai Nat in Thailand were investigated. Pictures of the sprouting mung beans are shown in Figure 18.

Figure 18: Intact mung beans (0h) and seeds germinated for 24, 36, 48, 60 and 75h of the cultivars Chai Nat 72 and Kam Phang Saen 2 grown at dry and rainy season.

The four materials were sprouted together under similar laboratory conditions to analyze the impact of genetic background and growing environment on the metabolites profiles during the sprouting process. Mung bean sprouting was conducted under conditions comparable to the germination procedure conducted in South East Asian countries following the scheme described in Materials and Methods. The lengths of the mung bean sprouts increased in the course of the germination process up to 9 cm after 75 hours of incubation.

Metabolite profiling data obtained for the two intact cultivars from the two different growing seasons revealed a differentiation between Kampang Saen 2 and Chai Nat 72. The metabolite profiling data were statistically assessed via principal component analysis demonstrating the clustering of the four mung beans samples. On the basis of the metabolite profiling data covered in the combined fractions I-IV, a clear separation according to the cultivars was observed on the first principal component

accounting for 42% of the variation, whereas a differentiation according to seasonal effects was observed along the second principal component (Figure 19). This clustering pattern indicates a less pronounced impact of the environmental growing conditions compared to the genetic influence.

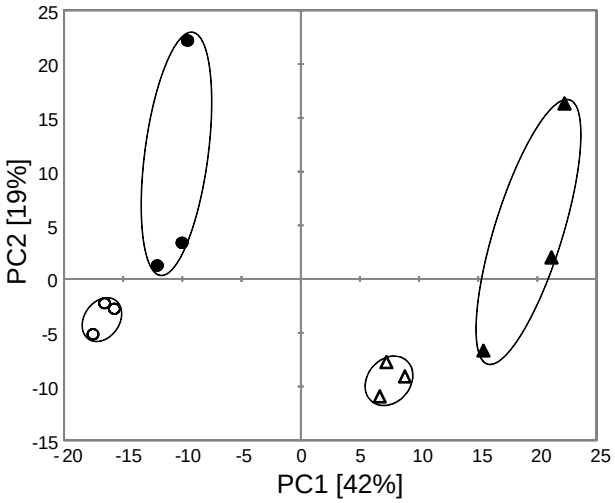


Figure 19: Principal component analysis of standardized GC/FID metabolite profiling data of the combined fractions I-IV from Kampang Saen 2 (●,O) and Chai Nat 72 (▲,Δ) grown at rainy (●,▲) and dry (O, Δ) season.

The two mung bean cultivars grown at rainy and dry seasons were subjected to the sprouting process. PCA plots with the metabolite profiling data of the combined fractions I-IV of Kampang Saen 2 and Chai Nat 72 are shown in Figure 20. The variation covered described 57 and 61%, respectively, for the two mung bean genotypes during sprouting. Both cultivars described a U-shape pattern during the incubation time. However, compared to the distinct differentiation according to incubation time, only minor differentiations were observed for the materials grown at dry and rainy seasons.

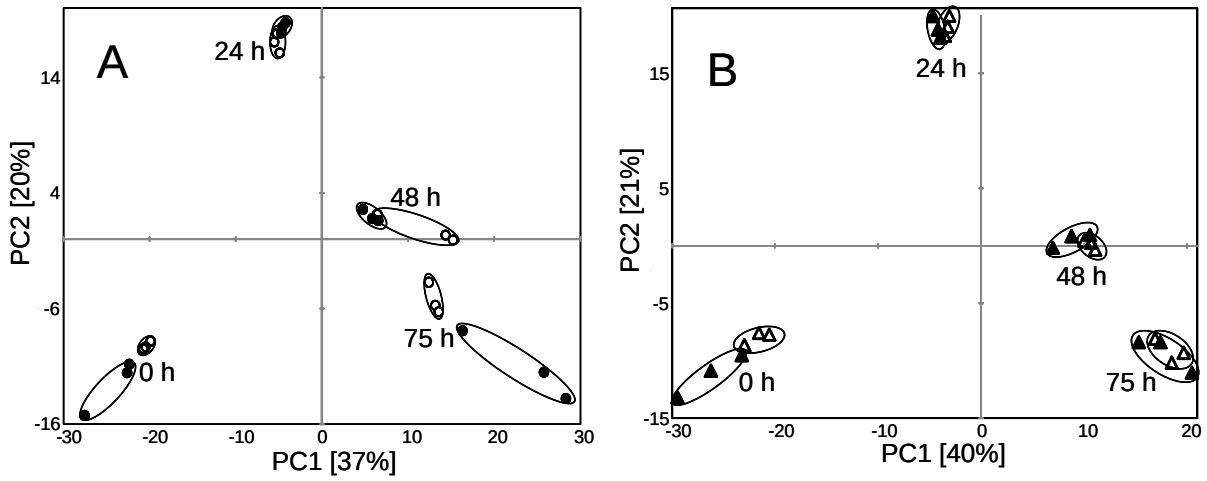


Figure 20: Principal component analysis (PCA) of standardized GC/FID metabolite profiling data of the combined fractions I-IV from rainy (●,▲) and dry (O, Δ) seasonal grown cultivars Kampang Saen 2 (A) and Chai Nat 72 (B) in the course of sprouting (0, 24, 48, 75h).

Principal component analyses containing the combined metabolite profiling data from both genotypes, i.e. Kampang Saen 2 and Chai Nat 72, are shown in Figure 21. The PCA plots demonstrated that the metabolic changes during the germination process are reflected by time-dependent shifts of the scores, which were similar for the four mung bean materials investigated. Compared to the factor genetics, the environmental impact on the metabolite profiles, i.e. growing season (dry, rainy), seems to be less pronounced in the course of the sprouting process. As example, in fraction III, sprouts germinated for 75h can be cleary separated between the two different genotypes (Figure 21D). On the other hand, for the single time points (24, 48, 75h), no clear differentiations were observed for the genotypes grown during dry and rainy seasons (Figure 21B-E).

On the basis of the metabolite profiling data, it was shown that the factor genotype is more pronounced than the seasonal differences during the mung bean growth. Although the factor environment (dry and rainy season) was shown to impact the metabolite profiles of intact mung beans (Figure 19), no significant differentiations in the metabolite profiles were observed for the sprouting mung beans according to this factor (Figure 21).

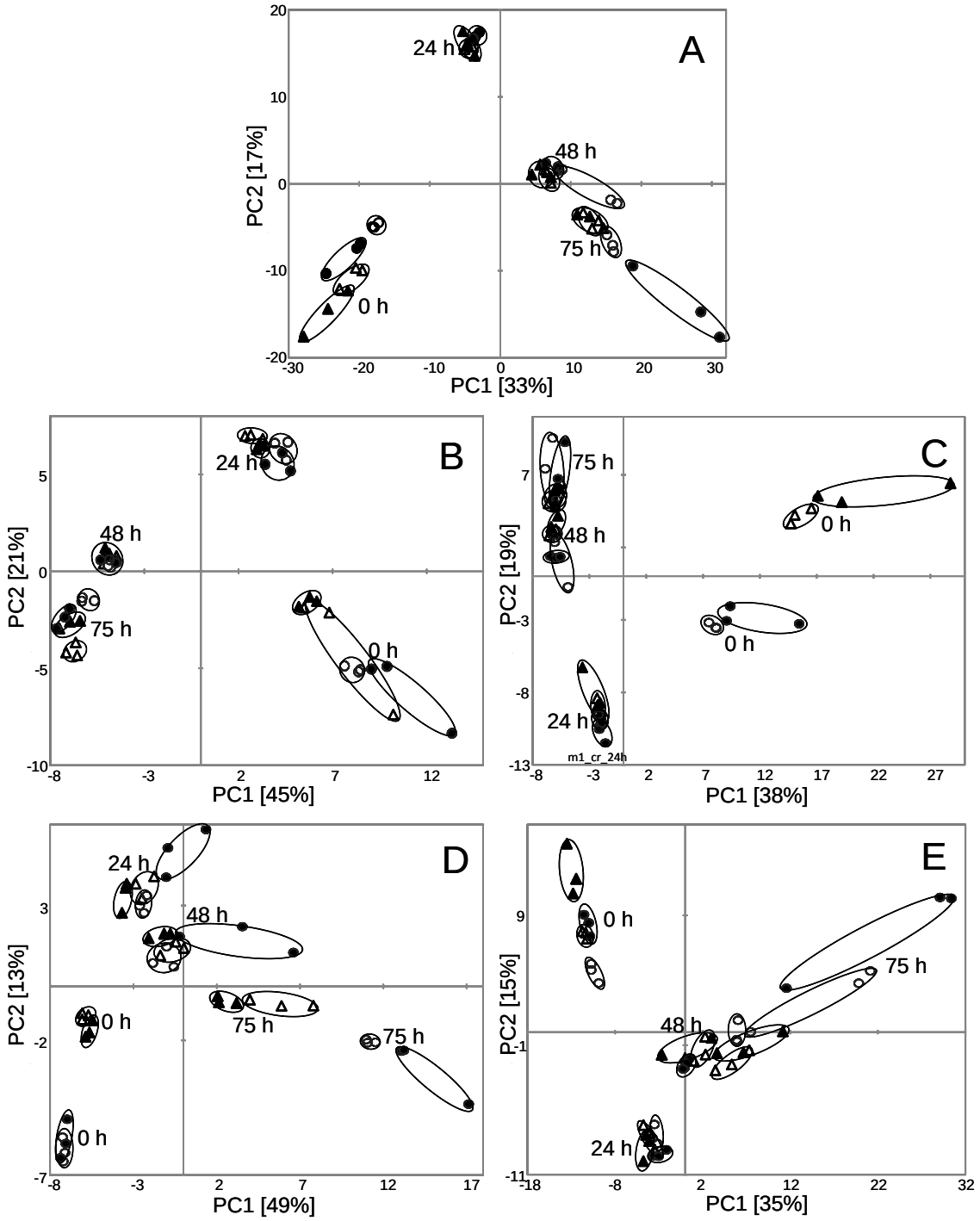


Figure 21: Principal component analysis (PCA) of standardized GC/FID metabolite profiling data of the combined fractions I-IV (A) and the single fractions I (B), II (C), III (D) and IV (E) from rainy (●,▲) and dry (O, Δ) seasonal grown cultivars Kampang Saen 2 (●,O) and Chai Nat 72 (▲,Δ) in the course of sprouting (0, 24, 48, 75h).

4.3.4 Influence of temperature

The incubation temperature has the most obvious influence on the seed sprouting progress. For industrial sprouting processes, the mung bean core temperatures depend on the adjusted ambient temperature and the amount of material to be germinated. For the home-made sprouting, incubation temperatures may vary greatly especially in Southern regions.

For the investigation of the influence of the incubation temperature, the sprouting of mung beans was conducted at 25°C and 35°C, respectively. Mung bean seeds were washed, subsequently soaked for 12 hours in tap water and sprouted at the given temperatures (Figure 22)

Figure 22: Sprouting mung bean seeds germinated at 25°C (A) and 35°C (B).

In nature, the germination of mung beans is epigeal (Mogotsi, 2006), which means that the extensions of the cotyledons are raised above the ground (Strasburger and Benecke, 1913). The cotyledons become green and are a certain time photosynthetically active (Kutschera, 2002). Within the laboratory germination at 35°C, the cotyledons were clearly visible after 48 h while at 25°C the cotyledons became visible not until 60 hours (Figure 22). In addition, the length of the hypocotyl, the section of the seminal root to the cotyledons, differed between the sprouts germinated at 25°C and 35°C, respectively. Samples were taken during the sprouting process and subjected to the metabolite profiling extraction and fractionation procedure as described in Materials and Methods. A principal component analysis containing the metabolite profiling data of the combined fractions I-IV from the same batch of mung bean materials incubated at the two different temperatures 25°C and 35°C is shown in Figure 23.

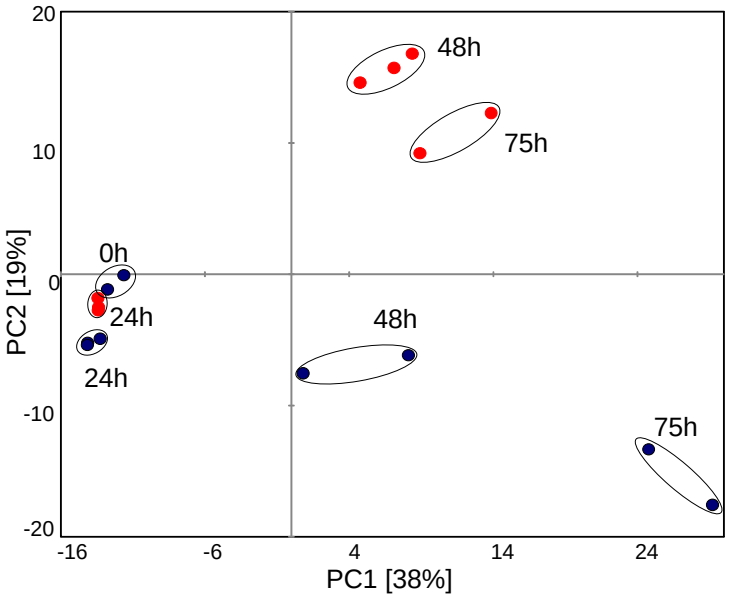


Figure 23: Principal component analysis of standardized GC metabolite profiling data of the combined fractions I-IV from the same batch of mung beans germinated at 25°C (●) and 35°C (●).

The metabolic changes are reflected by time-dependent shifts of the scores for the first two principal components PC1 and PC2. For both incubation temperatures, the patterns of the mung bean materials showed a drift along PC1. However, a differentiation according to the different temperatures was observed for the combined fractions especially along the second principal component after 48h (Figure 23); the

differences according to the temperature became more pronounced with increasing incubation times.

4.3.4.1 Fraction I

PCA scores plots illustrating the courses of sprouting mung beans incubated at different tempertures are shown in Figure 24. For the PCA showing the combined treatments (Figure 24A), a total variance of 66% was explainable by the first two PCs. For both temperatures (25°C and 35°C), the course of sprouting mung beans described a recumbent U-shape (Figures 24B-C). After an incubation time of 24 h, a left drift was observed along PC1. Mung beans sprouted at an ambient temperature of 35°C were separated from the samples incubated at 25°C along the second PC.

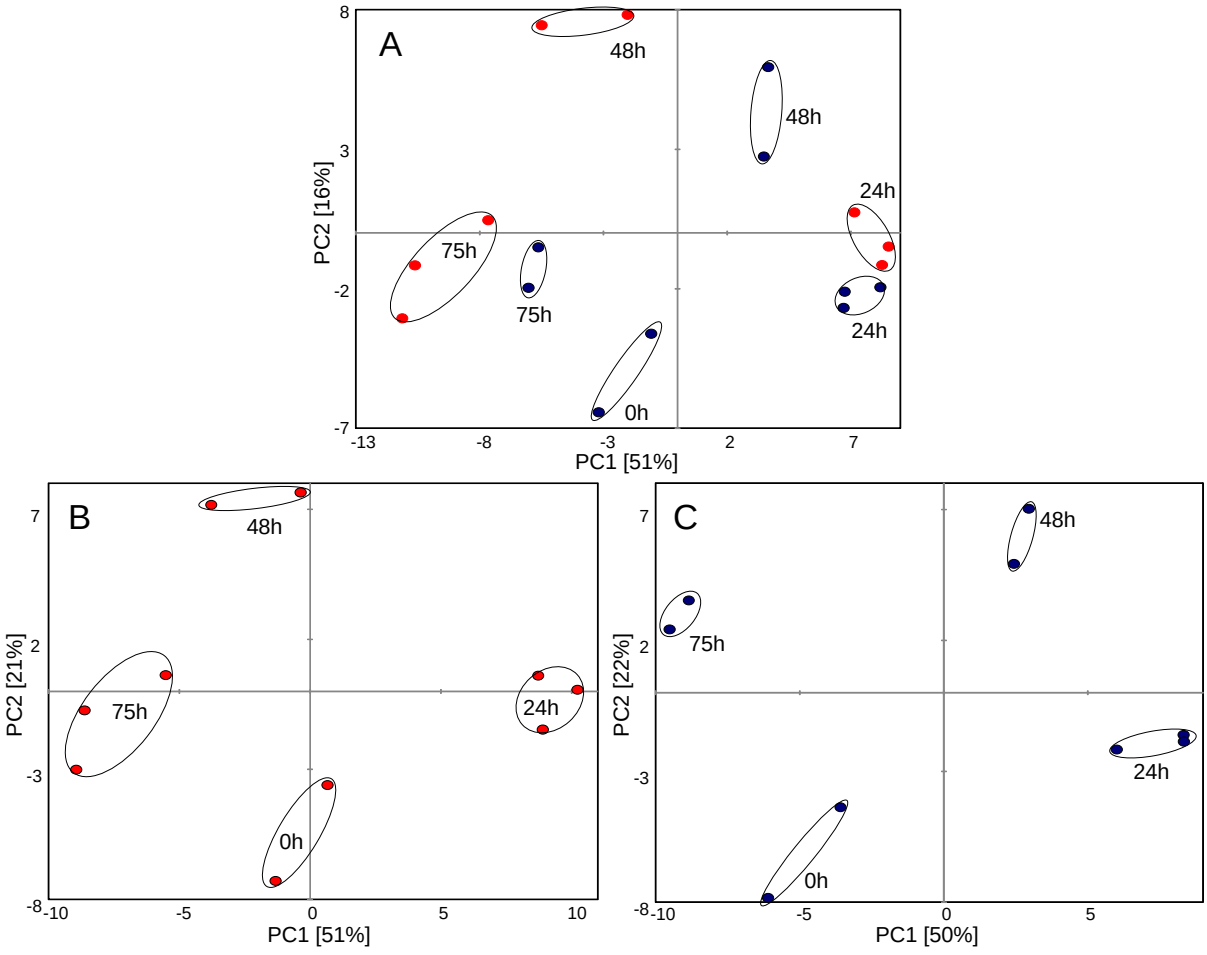


Figure 24: Principal component analysis of standardized GC metabolite profiling data of fraction I during the sprouting of mung beans at both temperatures (A) and separately at 35°C (B) and 25°C (C). ●, 25°C; ●, 35°C.

The dynamic changes of selected compounds from fraction I during the sprouting process are shown in Figure 25. For both saturated and unsaturated triglyceride-derived fatty acids, a uniform trend was observed. The highest levels of fatty acid methyl esters were determined after 24 hours of incubation. From 24h to 75h the FAMEs were found to continuously decrease.

Lipids are stored in legumes in the form of oil bodies, the oleosome. Already during the first hours of germination, they are made available for the growth of the seedlings. Therefore, decreased FAME contents during the sprouting progress are an expected result as triglycerides serve as major energy source for the developing embryo. For sprouting mung beans, decreased contents of total triglycerids have been described (Harmuth-Hoene et al., 1987; Abdel-Rahman et al., 2007). A comparable decrease in the contents of triglyceride-derived fatty acids has also been described for germinating rice (Shu et al., 2008) and barley seeds (Frank et al., 2011).

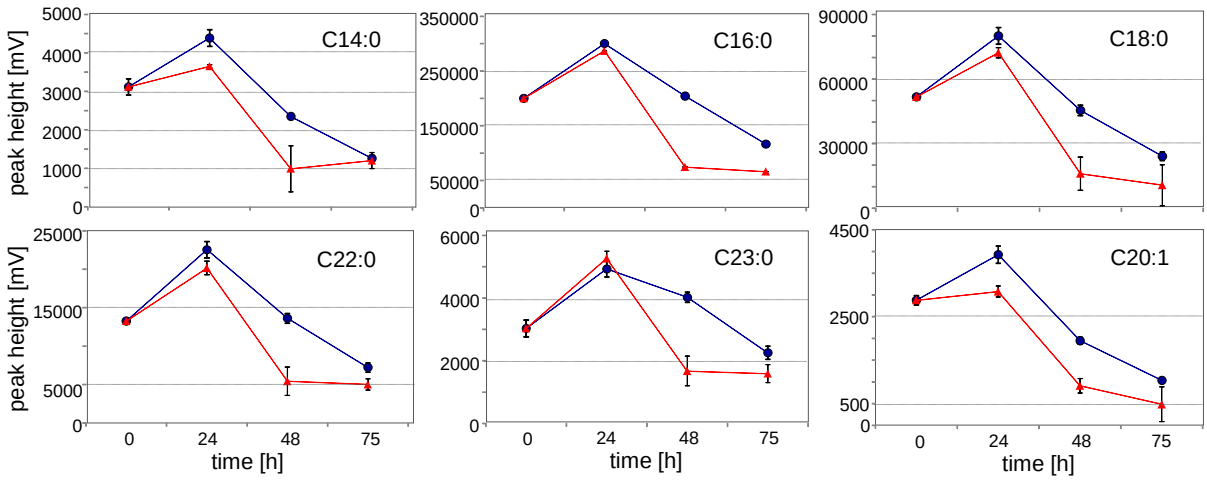


Figure 25: Changes of standardized peak heights for selected compounds in fraction I (saturated and unsaturated fatty acid methyl esters, hydrocarbon) during sprouting of mung beans at 25°C (•) and 35°C (•).

4.3.4.2 Fraction II

PCA scores plots illustrating the courses of sprouting mung beans incubated at different tempertures are shown in Figure 26. For the PCA showing the combined treatments (Figure 26A), a total variance of 55% can be explained by the first two PCs. The total variance explained by PC1 and PC2 is considerably lower than for the

other metabolite profiling fractions resulting in a less pronounced clustering of the mung beans germinated at 25°C and 35°C.

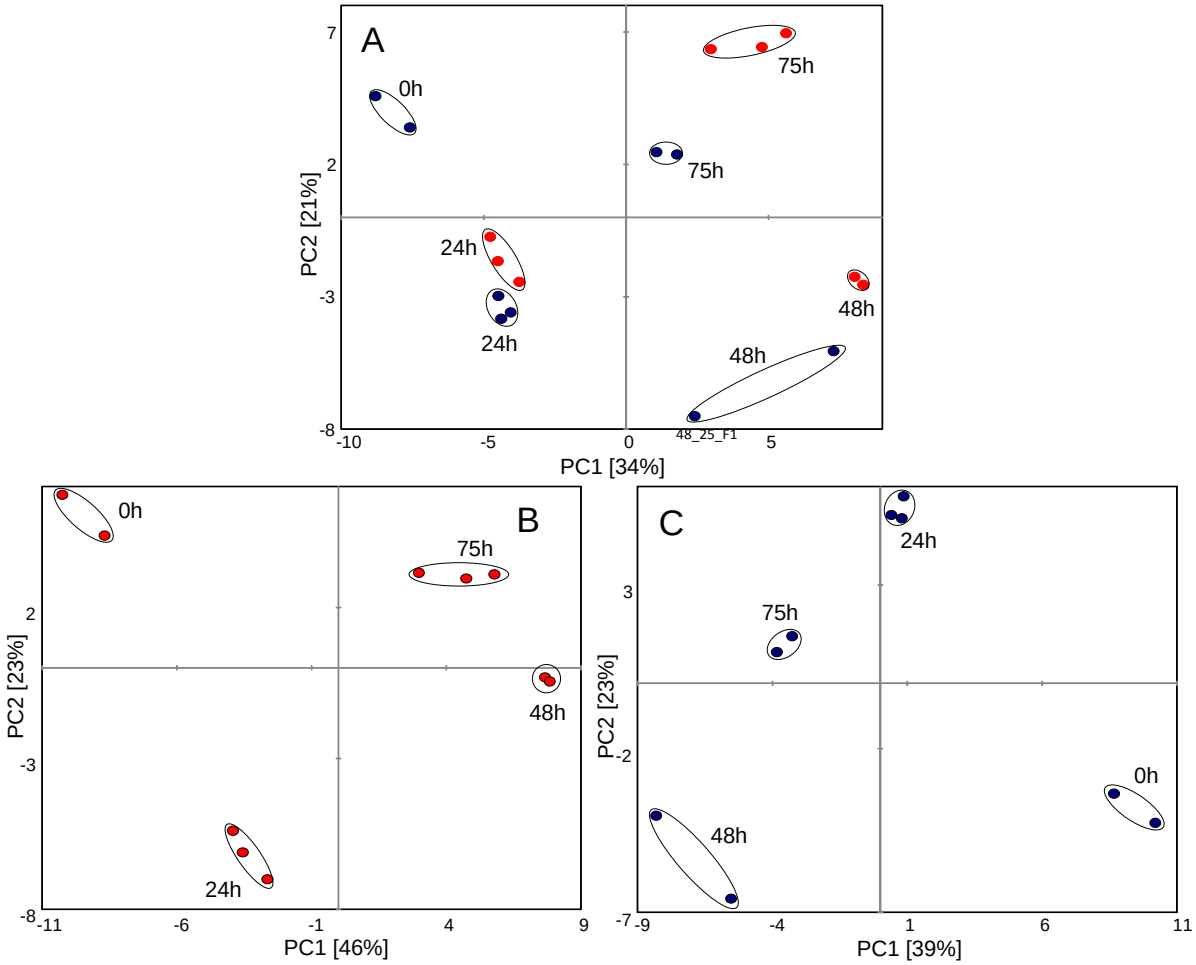


Figure 26: Principal component analysis of standardized GC metabolite profiling data of fraction II during the sprouting of mung beans at both temperatures (A) and separately at 35°C (B) and 25°C (C). ●, 25°C; ●, 35°C.

However, the samples incubated at 35°C could be slightly distinguished from the materials germinated at 25°C along PC2. Similar to the PCA plot containing the data from both germination temperatures, PCAs of material germinated at 25°C and the material germinated at 35°C showed undifferentiated clusters with regard to the sprouting process. This result indicates less pronounced metabolic changes in fraction II containing the minor lipids. Dynamic germination-time dependent changes of selected compounds from this fraction are shown in Figure 27. Overall, the observed changes were small. Except for a slight but continuous increase of sterols,

the profiles of free fatty acids, tocopherols and fatty alcohols showed an undifferentiated behaviour which is consistent with the results from the PCA scores. Increased sterol contents have been described for germinating mung beans by Kao (1935). In addition, a slight increase was also observed for rice in the course of germination (Shu et al., 2008).

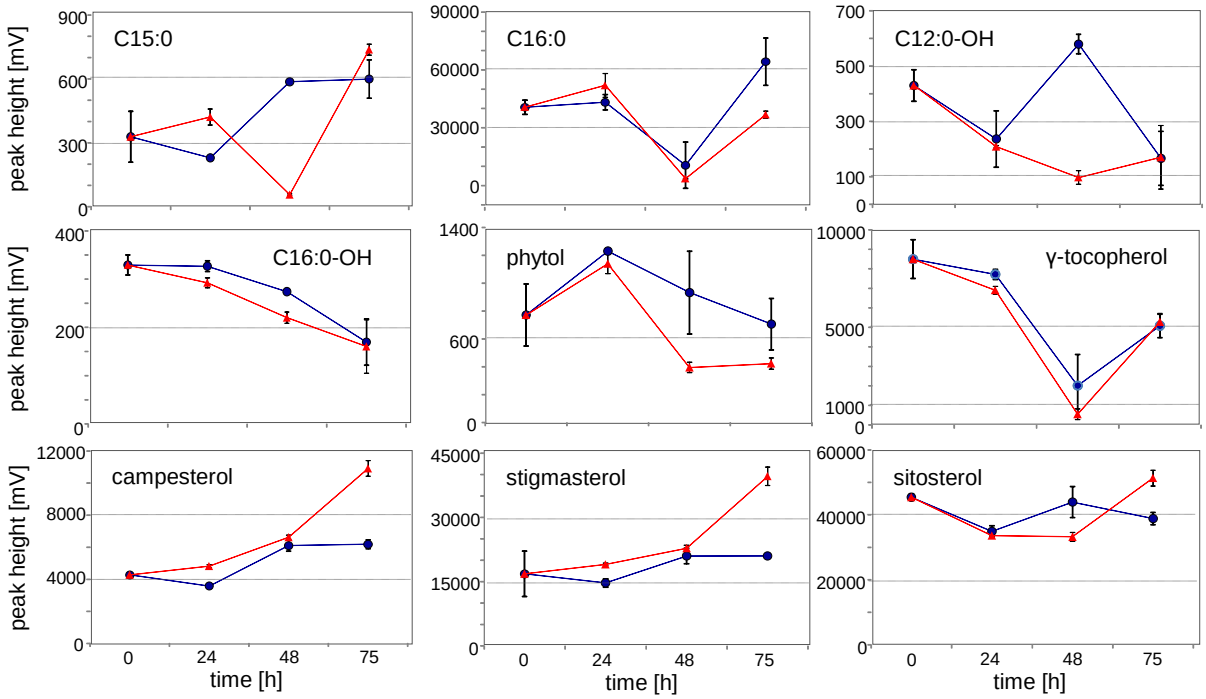


Figure 27: Changes of standardized peak heights for selected compounds in fraction II (minor lipids) during sprouting of mung beans at 25°C (●) and 35°C (●).

4.3.4.3 Fraction III

PCA scores plots illustrating the courses of sprouting mung beans incubated at the different tempertures are shown in Figure 28. For the PCA showing the combined treatments (Figure 28A), a total variance of 75% can be explained by the first two PCs.

Both mung beans germinated at 25°C and 35°C described a U-shape pattern in the course of sprouting. However, the clusters observed for the sprouting process at 35°C were right shifted compared to the germination at the lower temperature. For all two conditions (25°C and 35°C), the PCA scores plots indicate significant metabolic

changes during germination due to the clear clusterings at the latter sprouting stages (Figures 28B-C).

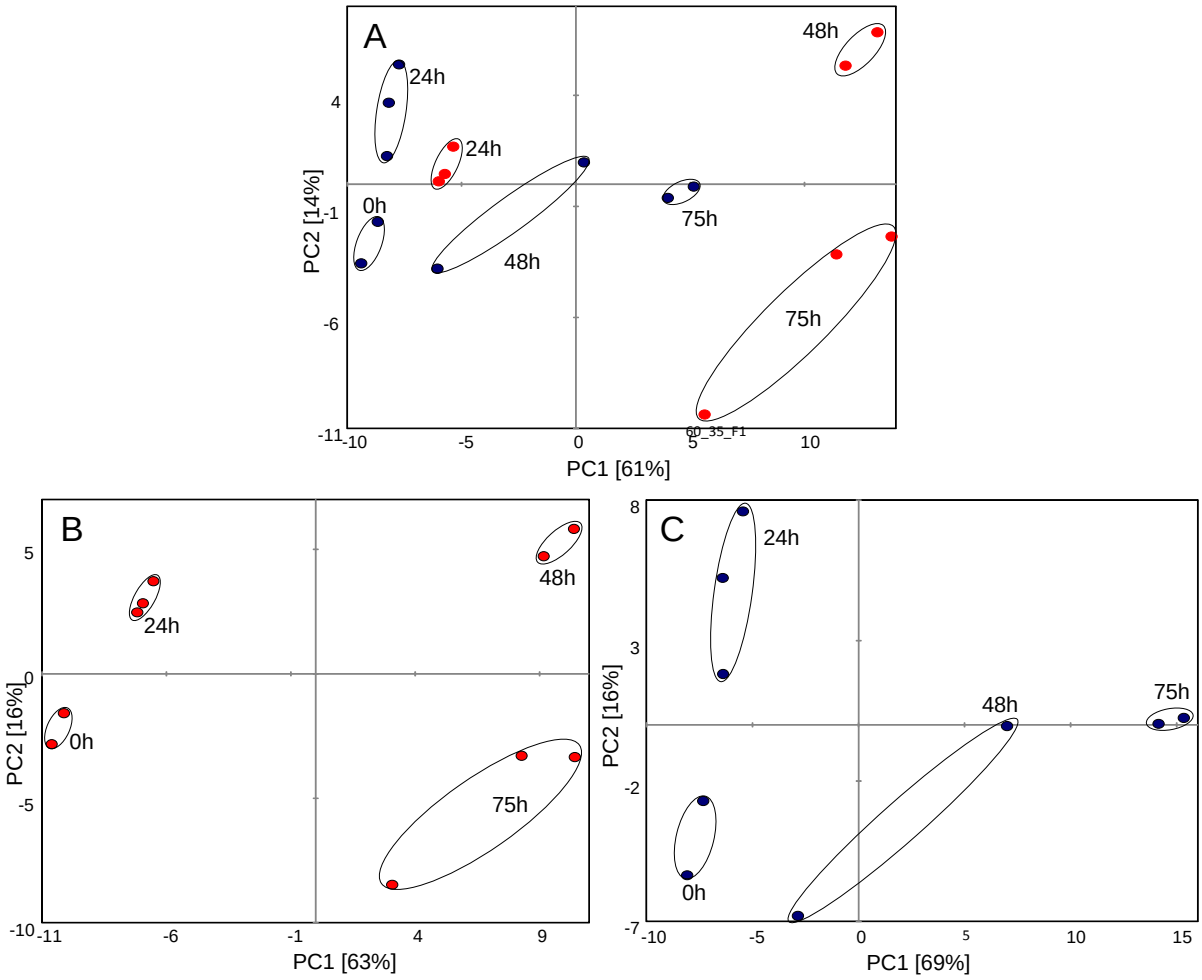


Figure 28: Principal component analysis of standardized GC metabolite profiling data of fraction III during the sprouting of mung beans at both temperatures (A) and separately at 35°C (B) and 25°C (C). ●, 25°C; ●, 35°C.

The dynamic changes of selected compounds from fraction III during the sprouting process are shown in Figure 29. A rapid increase for mono- and disaccharides has been observed after the initial soaking period (12h) for mono-and disaccharides; the increase at 35°C was much more pronounced than at 25°C.

Promila and Kumar (2000) found a low amylase activity within the first 18 hours of germination in mung beans, which could explain the delayed increase of glucose. The increased contents of mono- and disaccharides may reflect reductions in raffinose, stachyose and further oligosaccharides. In addition, during germination,

there occurs a breakdown of the starch, which accounts for the largest share of carbohydrate in ungerminated mung beans. Åman (1979) found similar curves for fructose, glucose, sucrose, raffinose and stachyose during germination of mung beans.

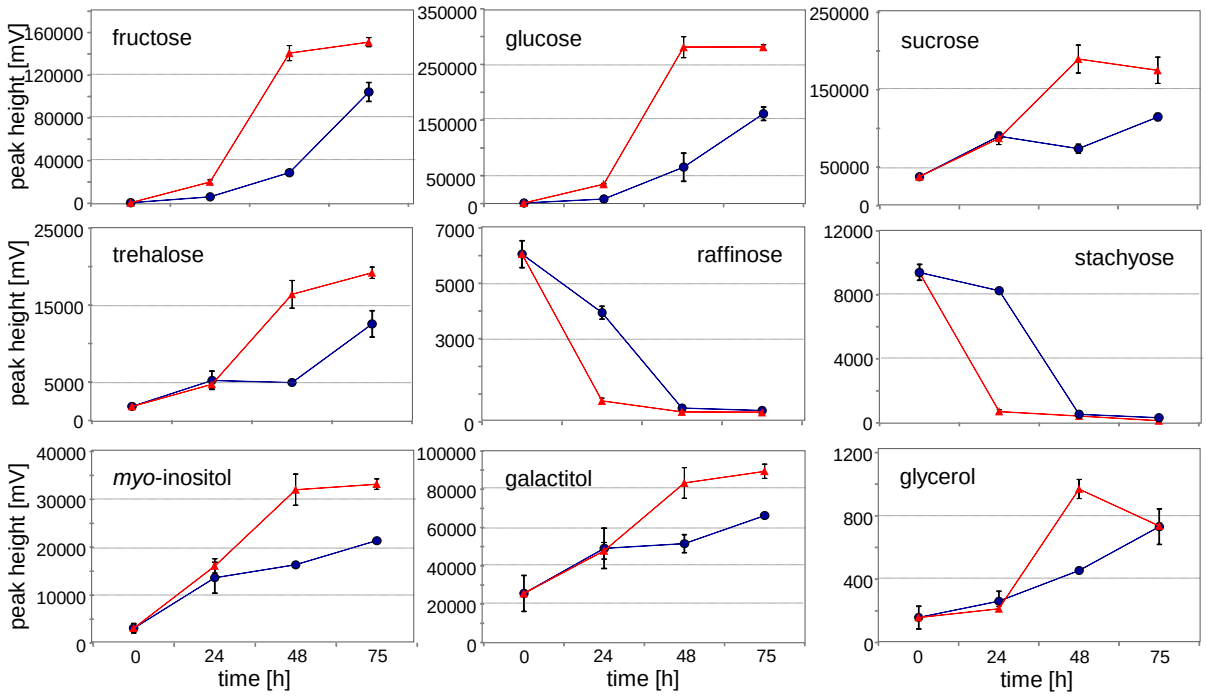


Figure 29: Changes of standardized peak heights for selected compounds in fraction III (sugars) during sprouting of mung beans at 25°C (•) and 35°C (•).

The contents of the sugar alcohol *myo*-inositol increased during incubation which indicates a decrease in the anti-nutrient phytic acid. Phytic acid is formed via phosphorylation of *myo*-inositol. During the sprouting of mung beans, phytic acid is degraded by enzymatic phytase activity which leads to enhanced levels of free *myo*-inositol. Until 48 hours of germination, this effect is more pronounced for the materials germinated at the higher temperature. However, at the subsequent time points, *myo*-inositol contents where shown to be at constant level which may indicate the change from catabolic and anabolic metabolism. As seen in Figure 22, mung bean sprouts germinated for 75h at 35°C formed already the roots indicating the change from germination to plant development.

4.3.4.4 Fraction IV

PCA scores plots illustrating the courses of sprouting mung beans incubated at different tempertures are shown in Figure 30.

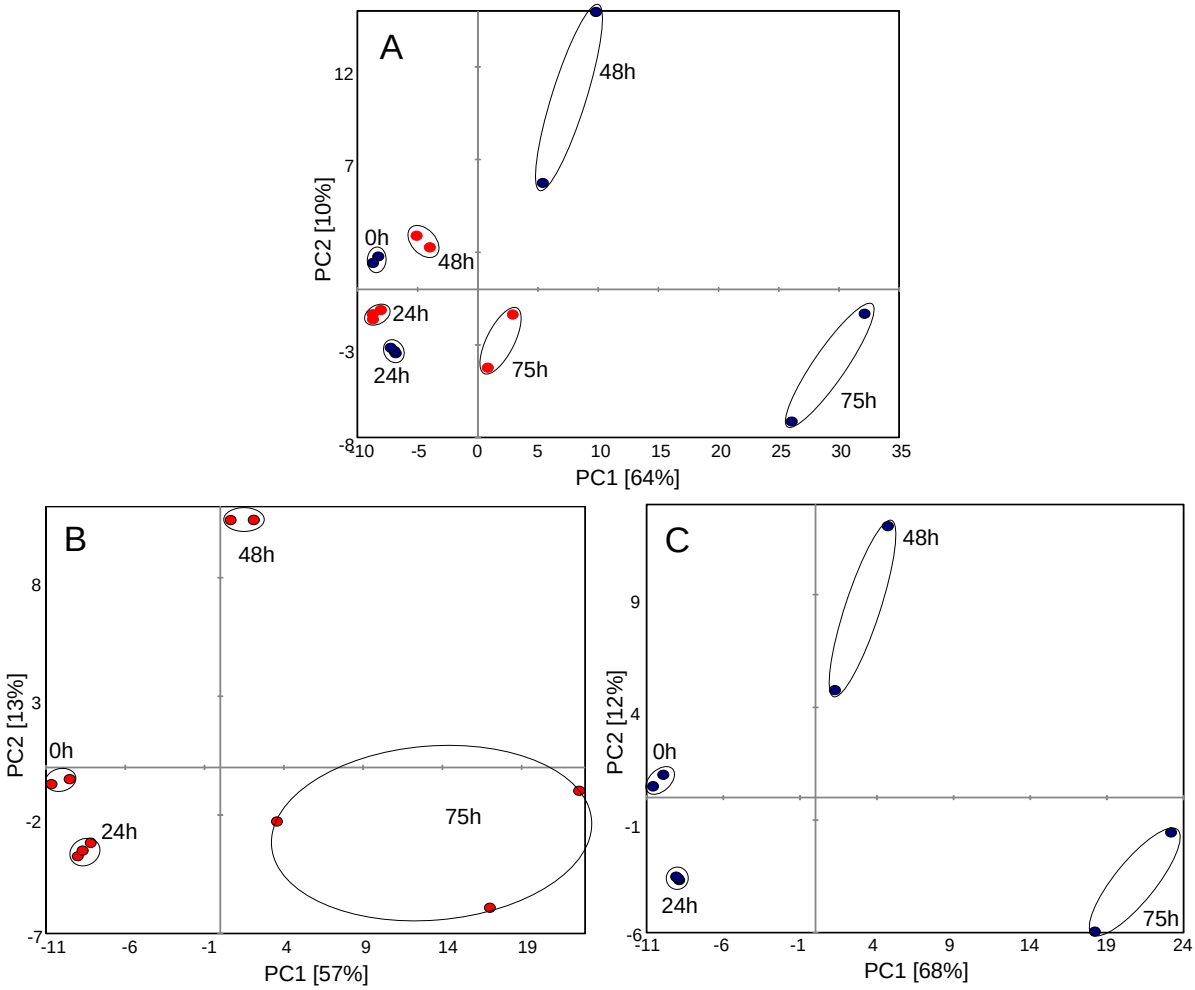


Figure 30: Principal component analysis of standardized GC metabolite profiling data of fraction IV during the sprouting of mung beans at both temperatures (A) and separately at 35°C (B) and 25°C (C). ●, 25°C; ●, 35°C.

For the PCA showing the combined treatments (Figure 30A), a total variance of 74% can be explained by the first two PCs. Similar to fraction III, mung bean germinated at 35°C exhibited a right shift along the first principal component compared to the materials sprouted at lower temperatures (25°C). Covering between 70% and 80% of the total metabolic variation, PCA scores revealed PC1 as the major component for

the germination time-dependent drift in the course of the sprouting progress (Figures 30B-C).

As expected, the increase of metabolites from fraction IV, i.e. inorganic and organic acids and amino acids was most pronounced in the mung beans germinated at 35°C (Figure 31). Generally, levels of acids were significantly increased after the initial soaking periods (12h). Morohashi and Shimokoriyama (1972) found also increasing contents of citric acid in germinating *Phaseolus mungo* beans. A significant increase in amino acid contents has been described for germinating rice (Shu et al., 2008). It was also noted, that the metabolic changes during the germination greatly depend on the respective incubation temperature.

In the course of germination, there is a proteolytic degradation of reserve proteins to poly- and dipeptides and free amino acids. Paul and Mukherjee (1972) observed a significant increase in protease activity until three hours after the start of germination in mung beans. The protein bodies of intact mung beans in the cotyledon contain reserve proteins as well as small amounts of peptidases, so that the proteolytic degradation can be initiated at an early stage of germination.

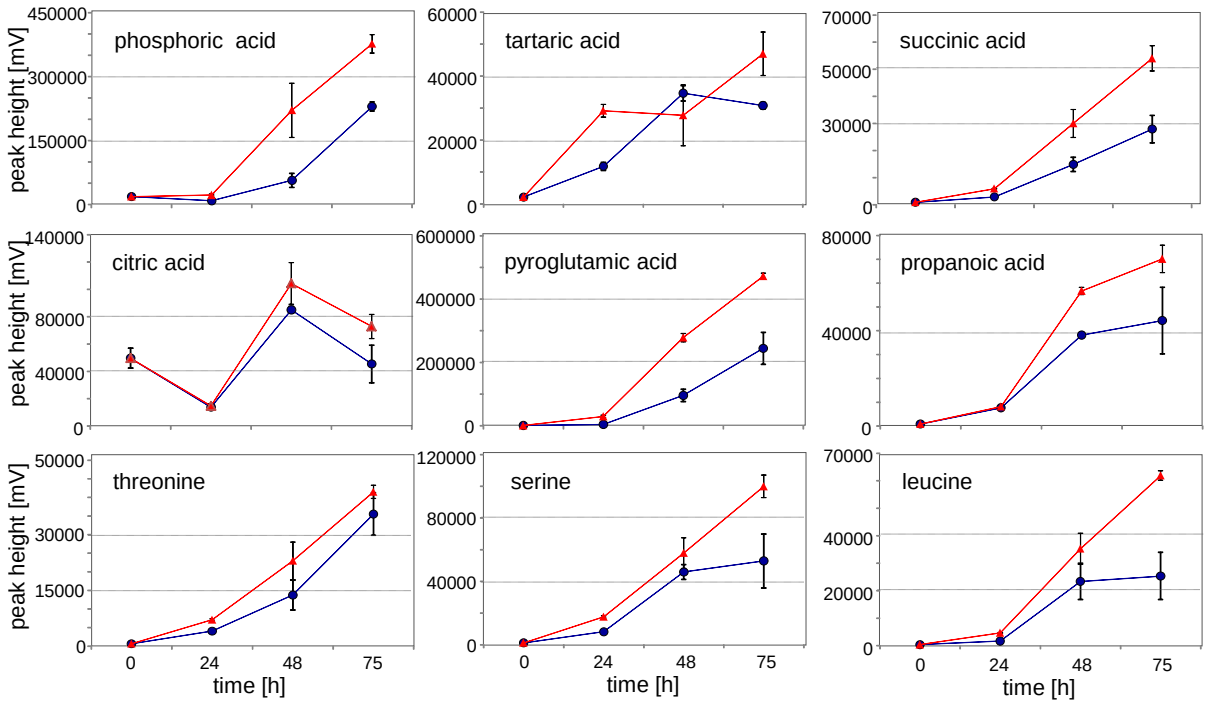


Figure 31: Changes of standardized peak heights for selected compounds in fraction IV (acids, amino acids) during sprouting of mung beans at 25°C (●) and 35°C (●).

4.3.4.5 Conclusions

The results obtained by the sprouting of mung beans at 25°C and 35°C confirm the considerable influence of the incubation temperature on the time-dependent metabolic changes. The mung beans sprouted at the higher temperature exhibited an accelerated metabolism resulting in metabolic changes at a very early stage of the sprouting process. The fast sprouting progress led to an early increase of nutritionally relevant metabolites, such as monosaccharides and free amino acids; whereas the levels of less desired tri- and tetrasaccharides were quickly reduced.

4.3.5 Influence of illumination

4.3.5.1 Illumination conditions during sprouting

Illumination by sun light is the basic requirement for plant development and photosynthesis. Light represents an important impact factor for the development of mung bean plants (Lawn and Ahn, 1985). An elevated light intensity was shown to result in higher yields of mung bean plants (Clifford, 1979).

The aim of the present study was to investigate the influence of illumination on the metabolite profiles of sprouting mung beans. The sprouting of mung beans was carried out under different conditions in an incubator. One batch was germinated completely in the dark and one batch was germinated under artificial light exposure. Mung beans were washed with tap water and soaked for 12 h at 30°C. They were then placed between moist filter paper in plastic trays in an incubator for germination. The light source for the germination under illumination was a 20 watt desk lamp, placed in the incubator. To prevent an overheating of the lamp, it was switched off every day for 1 hour. The temperature ranged between 28°C and 30°C in the incubator during the sprouting process. Germinating mung bean samples were kept permanently wet. After 24h, 48h and 72h, samples were taken and subjected to the metabolite profiling procedure. The sprouting progress of mung beans germinated under illumination (20W lamp) and under darkness is shown in Figure 32. The size of the seedlings increased constantly. After 24 hours, sprouts were on average 1.5 cm long and further increased from 3 cm to 9 cm after 48h and 72h. Interestingly, the sizes of the final sprouts germinated with and without illumination did not significantly differ (Figure 32).

The investigation of different light intensities of 50 W / m² and 150 W / m^2 to the field-based growth of mung beans revealed a more effective and productive growth for the plants illuminated with the brighter light source (Clifford, 1979). In the present study, the morphological differences after 24h of incubation were still low. The green seed coat was chapped and the radicle began to grow. After 48h the cotyledons were visible under both incubation conditions. The leaves of the sprouting mung beans germinated in the dark were yellow whereas they were green in the illumated materials. In addition, the hypocotyls (stem axis of the radicle to the cotyledons) were differently developed in color and aperture (Figure 32).

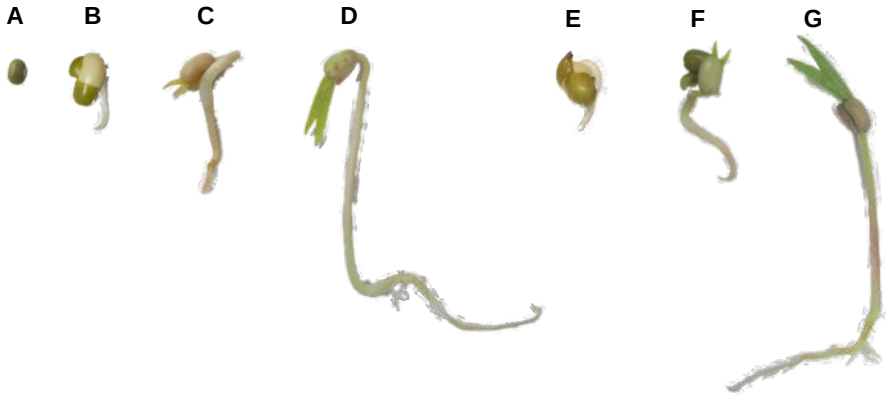


Figure 32: Mung bean (A) and mung bean sprouts germinated for 24h, 48h and 72 hours in the dark (B-D) and germinated under illumination by means of a 20 Watt lamp (E-G)

After a total of 72 hours a considerable extension of the hypocotyls for both approaches was observed. In addition, the growth of new root hairs indicated the end of the sole germination process and the beginning of an early plant growth phase. The cotyledons under the illumination approaches had obscured and a more green-purple color was recognizable. The hypocotyl was still thinner than for the sprouts germinated in the dark.

4.3.5.2 Fractions I-IV

The principal component analysis of the standardized GC/FID metabolite profiling data of the combined fractions I-IV in the course of mung bean sprouting is shown in Figure 33. The PCA scores plot revealed a distinct germination time-dependent right-drift along PC1. While the differences between 0h and 24h were relatively small, major separations were observed for the 48h and 72h samples. The PCA score confirms that the impact of the germination time is more pronounced than the impact of illumination. The time-dependent metabolic changes were mainly represented by the first principal component whereas the influence of illumination was mainly represented by PC2.

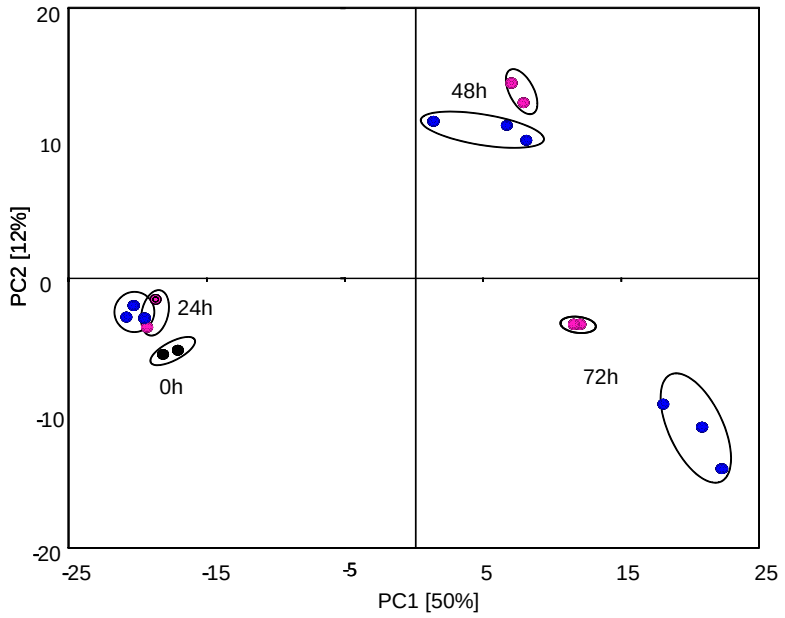


Figure 33: PCA of standardized GC metabolite profiling data of the combined fractions I-IV from mung beans during the germination process in the dark (●) and under illumination (●).

4.3.5.3 Fraction I

Figure 34 shows the PCA of fraction I during the germination process under illumination and darkness. The sprouting mung beans germinated for 48h and 72h could be clearly separated from the intact mung beans. However, compared to the clear time-dependent clustering of the mung beans, no differentiation was observed with regard to illumination (Figure 34).

The analysis of selected compounds from fraction I revealed that the metabolic variation observed between the sprouting mung beans under the influence of illumination was relatively small (Figure 35). A general decrease in total fat during germination has been described for mung beans in literature (Hartmuth-Hoene et al., 1987). The influence of light on germinating chickpea led to a higher fat content compared to chickpea germinated in the dark (Khattak et al., 2008a).

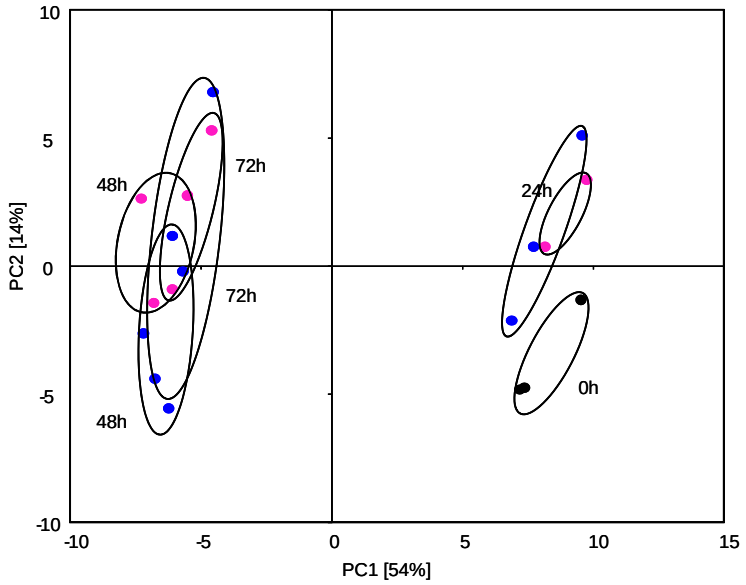


Figure 34: PCA of standardized GC metabolite profiling data of the combined fraction I from mung beans during the germination process in the dark (●) and under illumination (●).

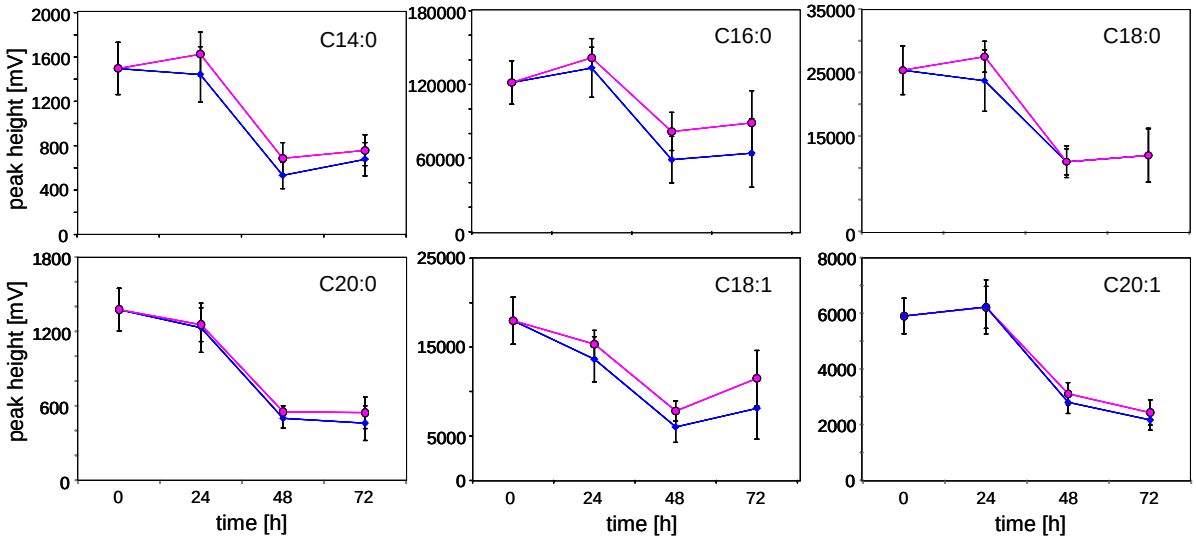


Figure 35: Changes of standardized peak heights for selected compounds in fraction I during the sprouting with (●) and without (●) illumination.

4.3.5.4 Fraction II

Figure 36 shows the PCA scores plot of fraction II during the germination process under illumination and darkness.

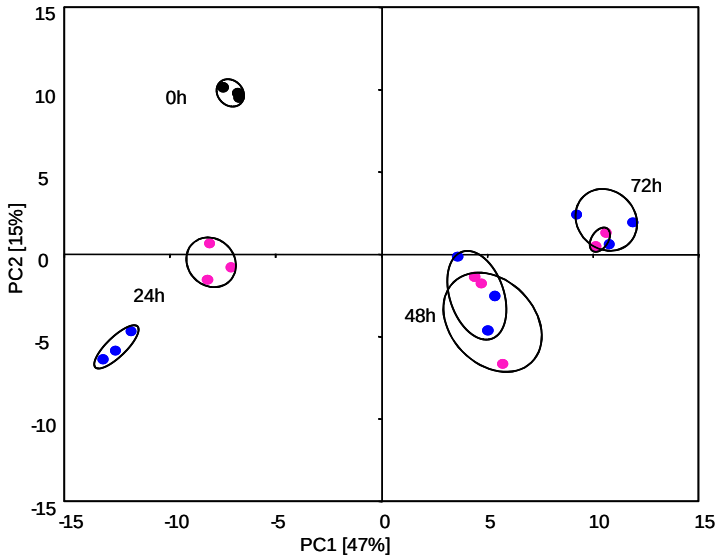


Figure 36: PCA of standardized GC metabolite profiling data of the combined fraction II from mung beans during the germination process in the dark (●) and under illumination (●).

Compared to the pronounced germination time-dependent metabolic changes observed in fraction II, the impact of illumination on the metabolite profiles of the sprouting mung beans seem rather low. Except for the samples germinated for 24h, the PCA revealed no separation of the samples sprouted with and without illumination (Figure 36).

Standardized peak heights for selected compounds in fraction II are shown in Figure 37. Increased sterol contents, e.g. of campesterol, stigmasterol and ß-sitosterol were observed for both incubation conditions (dark, light). A comparable alteration of sterol contents due to lighting as it was found within the growth of barley (Bush et al., 1971) was not observed in the present study. However, it has to be considered that changes in the concentrations of sterols during germination are strongly temperature-dependent. Lower sterol concentrations were found in plants grown at lower temperatures (Grunwald, 1975).

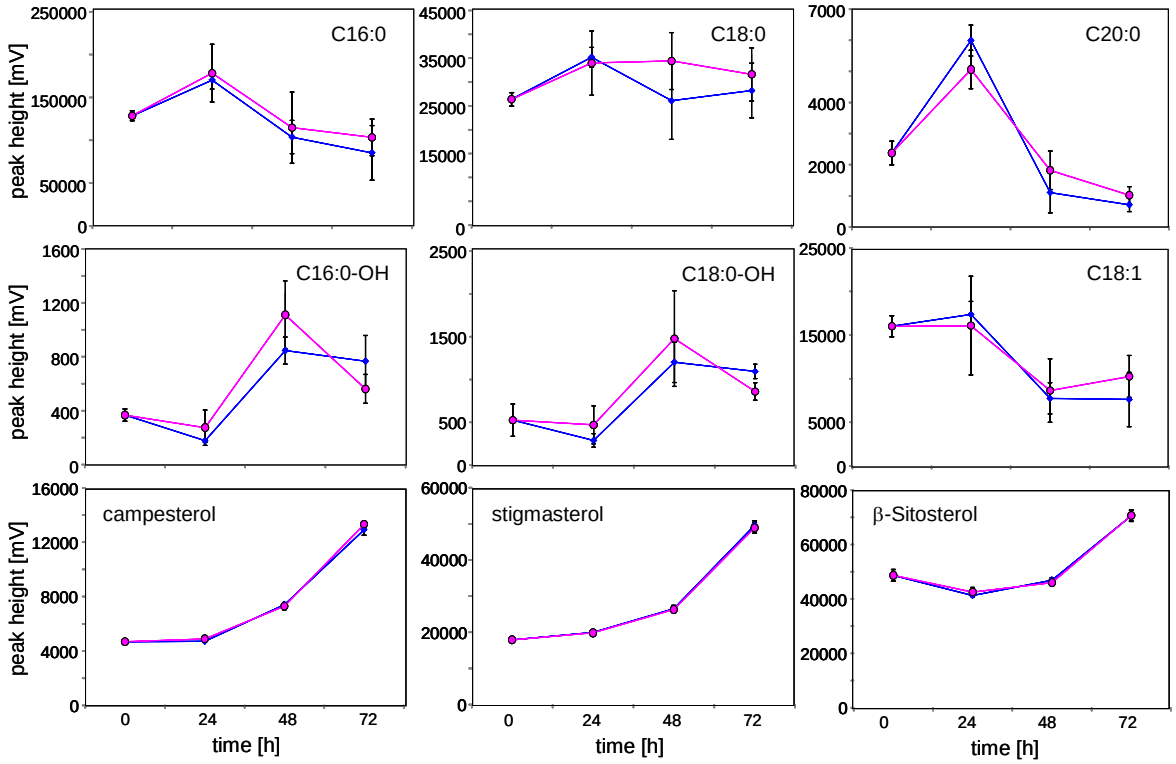


Figure 37: Changes of standardized peak heights for selected compounds in fraction II during the sprouting with (●) and without (●) illumination.

4.3.5.5 Fraction III

Figure 38 shows the PCA scores plots of fraction III during the germination process under illumination and darkness. The overall variation explained by PC1 and PC2 is 65%. The time-dependent clusters explained a U-shape pattern until the final incubation time. In contrast to the two non-polar fractions I and II, PCA of fraction III revealed cluster separations for illuminated germination *versus* dark germination. Until 24h of incubation, the clusters representing mung beans germinated in the dark and under illumination were close together. However, after 48h, the clusters began to drift along PC2. The final sprouts germinated for 72h could be separated on both principal components PC1 and PC2 (Figure 38).

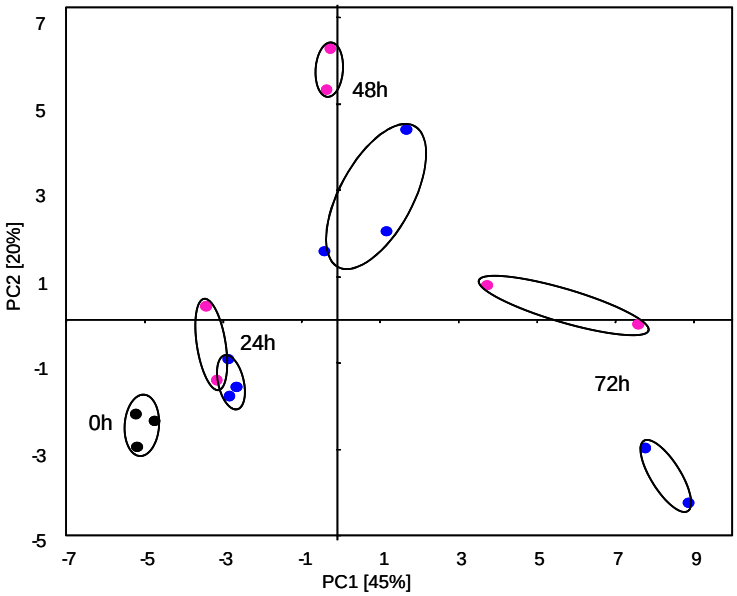


Figure 38: PCA of standardized GC metabolite profiling data of the combined fraction III from mung beans during the germination process in the dark (●) and under illumination (●).

Standardized peak heights for selected compounds in fraction III are shown in Figure 39. In accordance to the multivariate analysis, univariate assessment revealed differences for the illumination conditions.

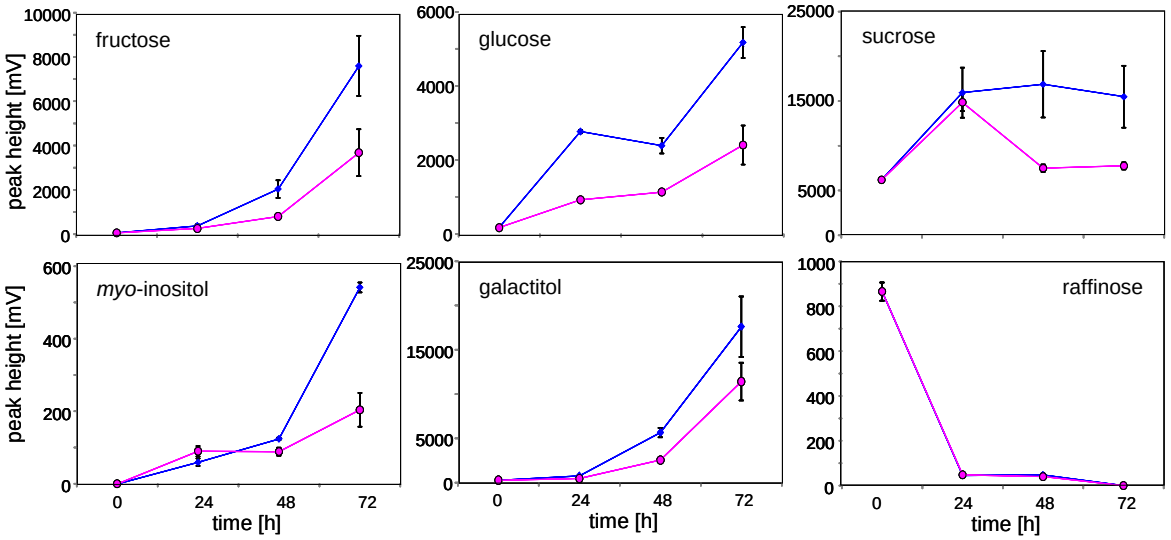


Figure 39: Changes of standardized peak heights for selected compounds in fraction III during the sprouting with (●) and without (●) illumination.

Interestingly, sprouting of mung beans in the dark led to stronger metabolic changes compared to the illuminated materials. This means that sprouts germinated without illumination contained higher contents of monosaccharides and sugar alcohols (Figure 39). In a comparable study on the influence of illumination during the germination of soybeans, no significant differences were found in the contents of free sugars (Choi and Bajpai, 2010). However, another study revealed higher sugar contents in germinating sunflower seeds when the seedlings were illuminated (Darbelley et al., 1997). The effect was explained by an increased α-amylase activity. Increased levels of monosaccharides, e.g. fructose and glucose and decreased levels of the tri- and tetrasaccharides raffinose and stachyose are to be expected during germination (Lumpkin and McClary, 1994, Shu et al, 2008).

4.3.5.6 Fraction IV

Figure 40 shows the PCA scores plots of fraction IV during the germination process under illumination and darkness.

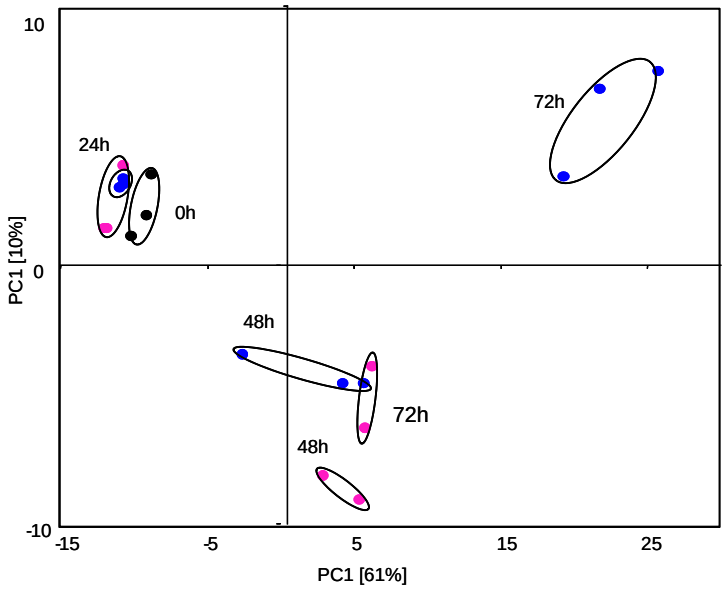


Figure 40: PCA of standardized GC metabolite profiling data of the combined fraction IV from mung beans during the germination process in the dark (●) and under illumination (●).

Similar to fraction III, after a total incubation time of 48h, the PCA scores plot exhibited a clustering for the illuminated and non-illuminated mung bean sprouts. The final sprouts germinated for 72h could be clearly separated on both principal components PC1 and PC2 (Figure 40).

Standardized peak heights for selected compounds in fraction IV are shown in Figure 41. Mung bean sprouts germinated in the dark contained higher levels of acids and free amino acids than the sprouts germinated under illumination. In contrast to the present study, Choi and Bajpai (2010) found no significant differences in amino acid levels of soybeans germinated with and without illumination. In addition, illumination has been shown to result also in increased contents of free amino acids in germinating beans (Kuo et al., 2003) and chickpea especially for the treatments with γ-irradiation, fluorescence and yellow light (Khattak et al., 2008b).

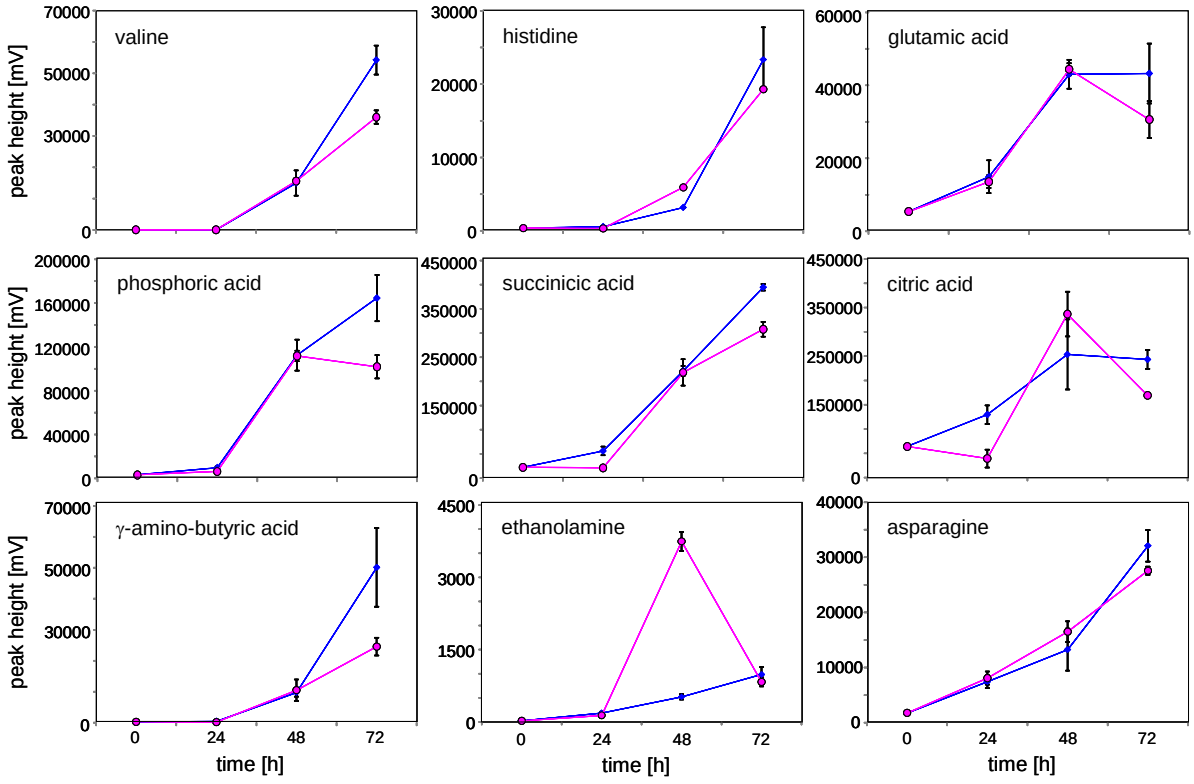


Figure 41: Changes of standardized peak heights for selected compounds in fraction IV during the sprouting with (●) and without (●) illumination.

4.3.6 Impact of washing

Mung bean sprouts are a widely available food which can be purchased as “ready-to-eat” product or may be consumed after germinating the mung beans at home (Fery, 2002). Consumable raw sprouts obtained from legumes and cereals may constitute a potential hazard with respect to microbial contaminations. For mung beans, several microbial infections have been reported in conjunction with the consumption of raw sprouts (Mohle-Boetani et al., 2009). Different disinfection procedures, e.g. chemical sanitation (Munoz et al., 2006), dry heat and irradiation (Bari et al., 2009) have been tested for their suitability for seed treatments before and after sprouting. However, it has to be kept in mind that the practical application of most preservation treatments is restricted by regulations. For example, only few organic acids including citric acid are allowed in the EU to be used for preservation treatments of organically grown and produced raw ready-to-eat sprouts (EG 2092/91; EG 834/2007; EG 889/2008).
The aim of the present study was to investigate the influence of washing steps of mung bean seeds prior and during the sprouting process. Millipore water and methanol were used as washing agents on a laboratory scale. In addition, the impact of a industrial-scale mung bean sprouting washing step with citric acid-containing water was investigated.

4.3.6.1 Laboratory-produced sprouts

Mung bean seeds obtained from a local health-food shop were washed and subsequently soaked for 12 hours in tap water prior to germination at an ambient temperature of 25°C. In addition, seeds from the same batch were washed with Millipore water instead of tap water and treated with methanol before soaking and incubation. Both mung bean seeds were subjected together to the sprouting procedure. Figure 42 shows the morphological changes in the course of germination of the differently treated mung beans incubated at 25°C.

Figure 42: Sprouting mung beans after washing the intact seeds with Millipore water and methanol (A) and tap water (B). Incubation temperature was set to 25°C.

Differences in morphology, e.g. sprout length, were not observed between the samples with and withouth the washing procedure with Milipore water and methanol. Samples were taken during the sprouting process at specific time points until 75 hours of incubation time and subjected to the metabolite profiling extraction and fractionation procedure described in Materials and Methods. PCA scores plots indicating the courses of sprouting mung beans treated with different washing conditions are shown in Figure 43. For the PCA showing the data of fractions I-IV for both treatments (seeds washed with Millipore water and methanol *versus* washing in tap water), a total variance of 58% was explainable by the first two PCs (Figure 43A). For both treatments, the course of sprouting mung beans followed a recumbent U-shape.

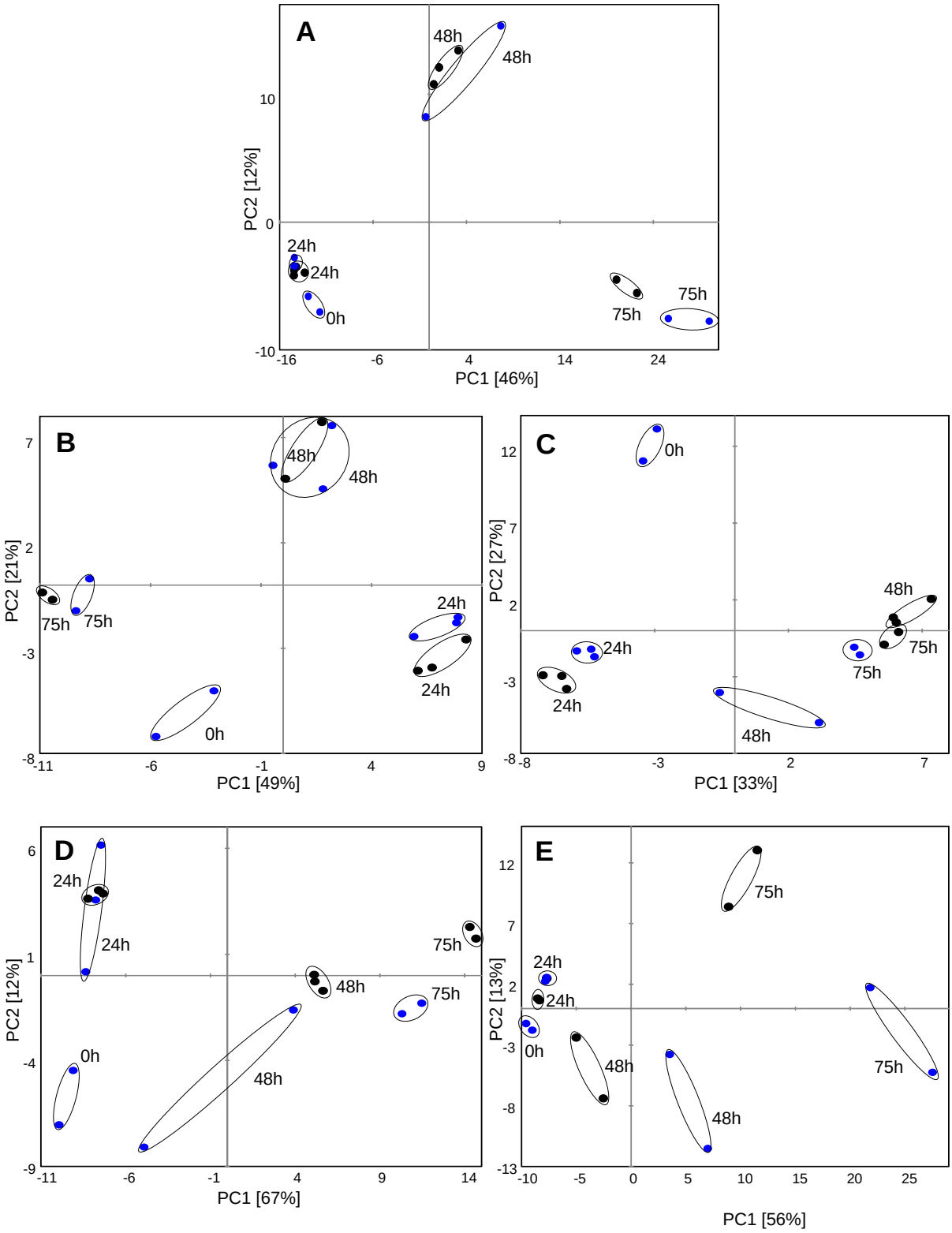


Figure 43: Principal component analysis (PCA) of standardized GC metabolite profiling data of the combined fractions I-IV (A) and the single fractions I (B), II (C), III (D) and IV (E) from the Millipore- and methanol-washed (●) and tap water-washed (●) mung beans germinated at 25°C.

Sprouting washed and non-washed mung beans showed comparable curves within the scores plots of the non-polar fractions I and II, whereas the clusters representing the polar constituents could be separated with respect to the washing treatment especially in the latter stages of germination (Figure 43). Univariate analyses of selected compounds from the treated and non-treated materials during the sprouting process are shown in Figures 44 and 45.

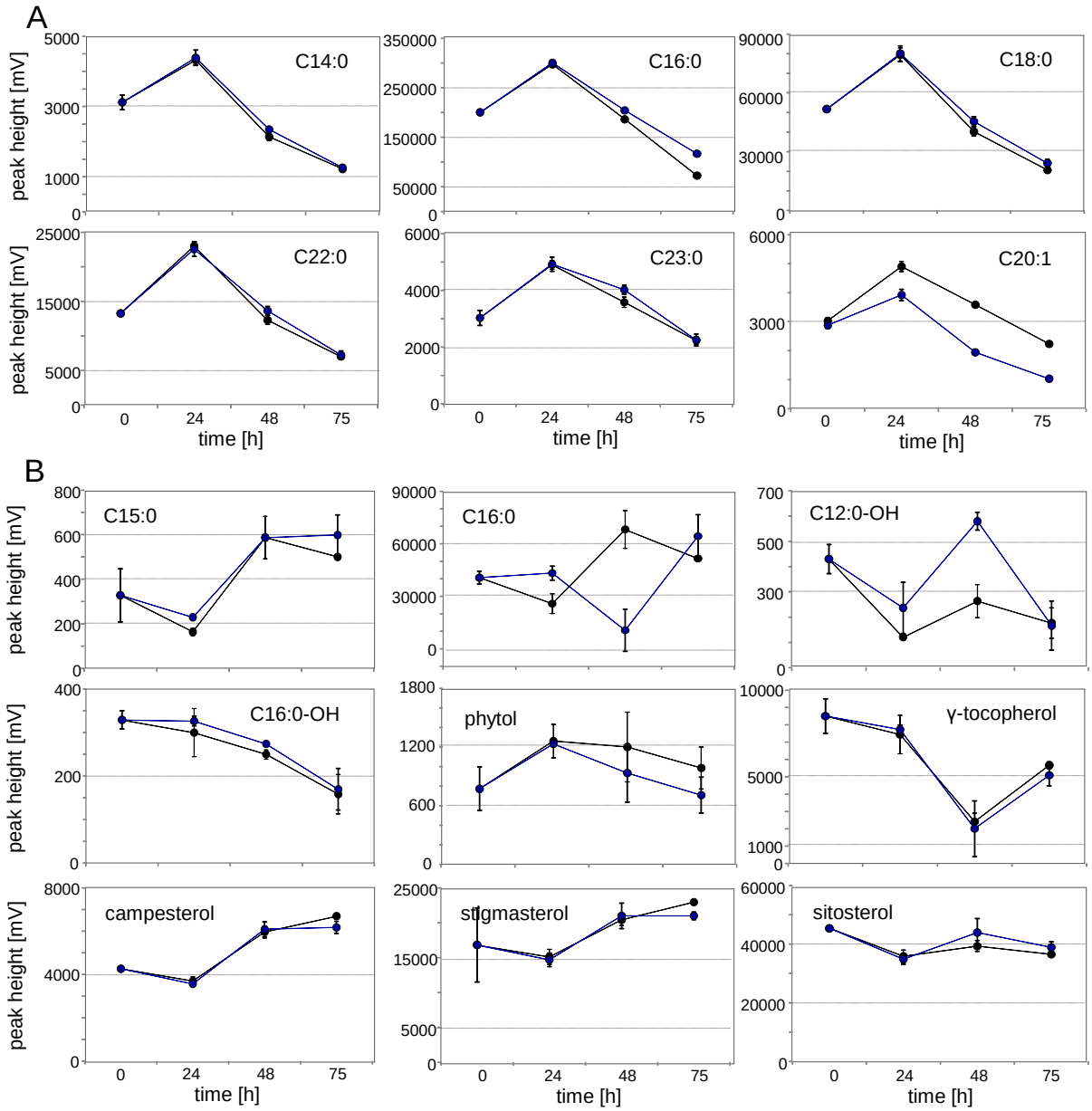


Figure 44: Changes of standardized peak heights for selected compounds in fractions I (A) and II (B) during sprouting of Millipore-and methanol-washed (●) and tap water-washed (●) mung beans at 25°C.

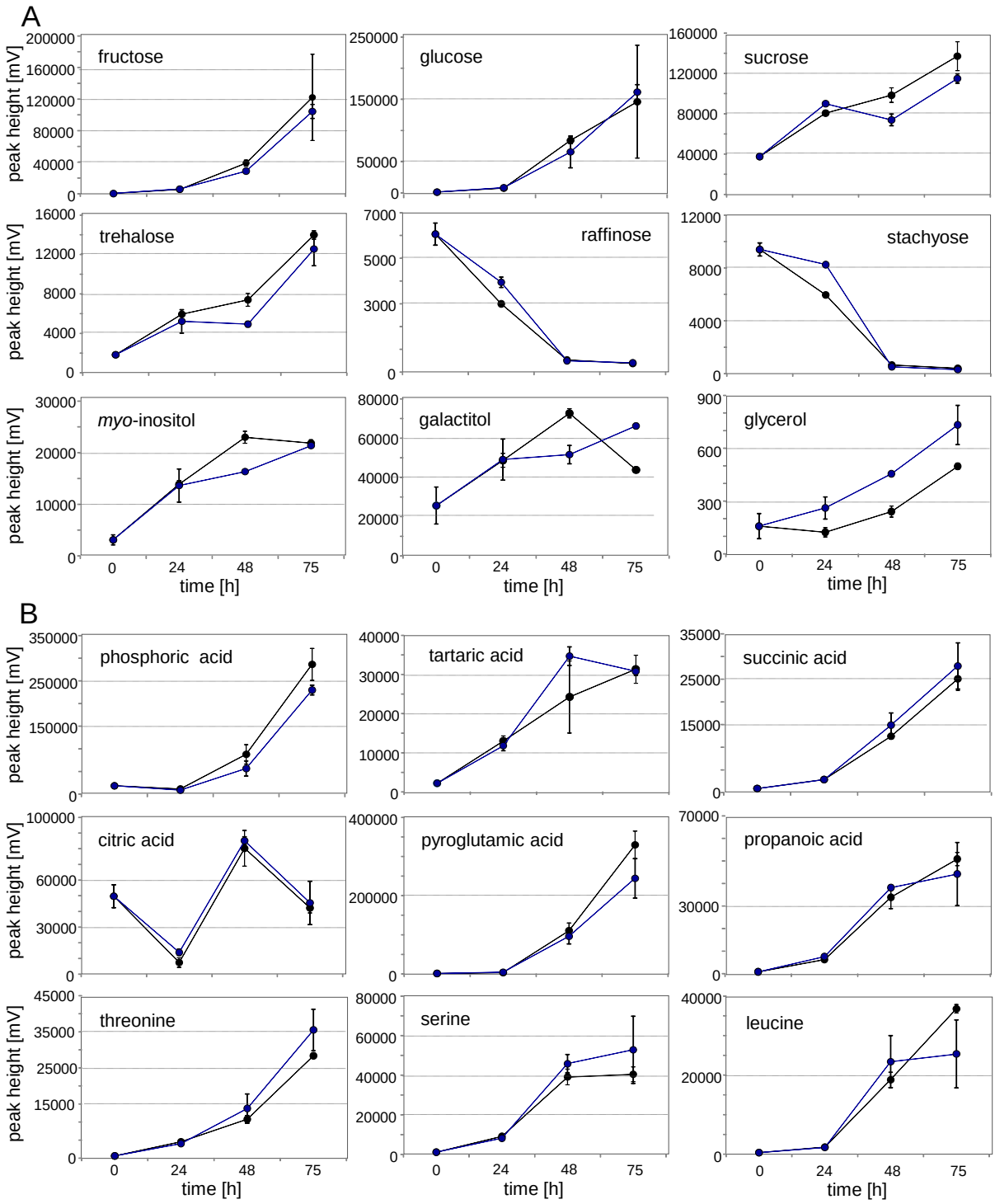


Figure 45: Changes of standardized peak heights for selected compounds in fractions III (A) and IV (B) during sprouting of Millipore-and methanol-washed (●) and tap water-washed (●) mung beans at 25°C.

The dynamic changes for the compounds during the sprouting of mung beans were accompanied by a continuous decrease in FAMEs, whereas the observed changes in fraction II representing the minor lipids were small. Significant increases in mono-

and disaccharides as well as in the levels of acids in fraction IV were observed. However, except for some amino acids, e.g. serine and leucine, no obvious differences between the Millipore water and methanol-treated and non-treated mung beans were detected. This indicates that the effect of Millipore-washing and methanol-treatment (prior to germination) on the metabolite profiles is rather low compared to the considerable germination time-dependent metabolic changes.

4.3.6.2 Commercially produced sprouts

The impact of an industrial-scale mung bean sprouting including a washing step with citric acid-containing water should be investigated compared to the same batch of mung beans sprouted without such a treatment. Intact mung beans and sprouting seeds, taken at different time points in the course of an industrial sprouting process (0h and after 24, 48 and 72h), were obtained by a commercial sprout producer. Germinated seeds, so called “mini-mung bean sprouts” (24h) and mung bean sprouts (72h) were received as non-washed (In) and washed (Iw) raw material. The washing steps consisted of a treatment with citric acid-containing tap water (pH 2.8-2.9) for preservation. The developmental changes observed in the course of the sprouting progress of the industrially produced sprouts are shown in Figure 46. Compared to the non-washed industrial sprouts, the washed sprouts exhibited a brighter surface.

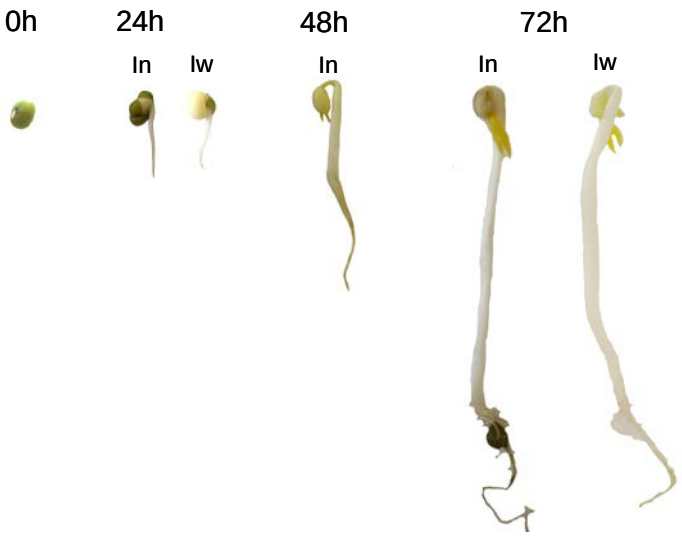


Figure 46: Intact mung beans (0h) obtained from industry and their seeds germinated for 24h, 48h and 72h under industrial conditions. In, non-washed, Iw washed.

For the comparative investigation of metabolite profiles from sprouting mung beans obtained by industrial germination procedures, samples were taken at the different time points, and their metabolite profiles were subjected to multivariate analysis. Differences in the metabolite profiles of washed versus non-washed germinated sprouts are reflected by the PCA scores plots of the combined and single non-polar and polar fractions I-IV shown in Figure 47. The scores plot containing the data from fraction I-IV (Figure 47A) revealed a clear separation of the citric acid-treated mini-mung bean sprouts and final sprouts from the non-treated materials after 24h and 72h of germination. Fractions I and II exhibited distinct clusters for the non-washed and washed sprouts after 24h (Figure 47B and C), whereas fractions III and IV showed distinct clusters for the sprouts germinated for 72h (Figure 47D and E).

Non-polar fractions

The changes of selected non-polar metabolites in the course of the sprouting processes are shown on the basis on standardized peak heights in Figure 48. The triglyceride-derived fatty acids (represented as fatty acid methyl esters, FAME) were shown to be decreased in the sprouts compared to the intact seeds (Figure 48A). The so called “mini-mung bean sprouts” washed after 24h of germination with citric acid-containing tap water exhibited significantly lower FAME contents compared to the non-washed industrial counterpart which led to the distinct clustering seen in Figure 47B. This result raises the need for additional trials on the effect of a washing treatment of sprouts with citric acid as a decrease in FAME contents through the conducted washing procedure was rather unexpected.

Changes in the levels of metabolites from fraction II, representing the minor lipids, were not consistently pronounced (Figure 48B). The changes reported for free fatty acids during the germination of crops are not consistent (Shu et al., 2008; Frank et al., 2011). However, it should be noted that the sprouting conditions have a great impact in particular for this compound class. No consistent trend was observed for the quantitative changes of fatty alcohols during the whole sprouting procedure. The quantitative course of γ-tocopherol, the major tocopherol in mung bean seeds, described a U-shape pattern during the laboratory germination. Although an increase of α-tocopherol was observed during sprouting, its total level remained low.

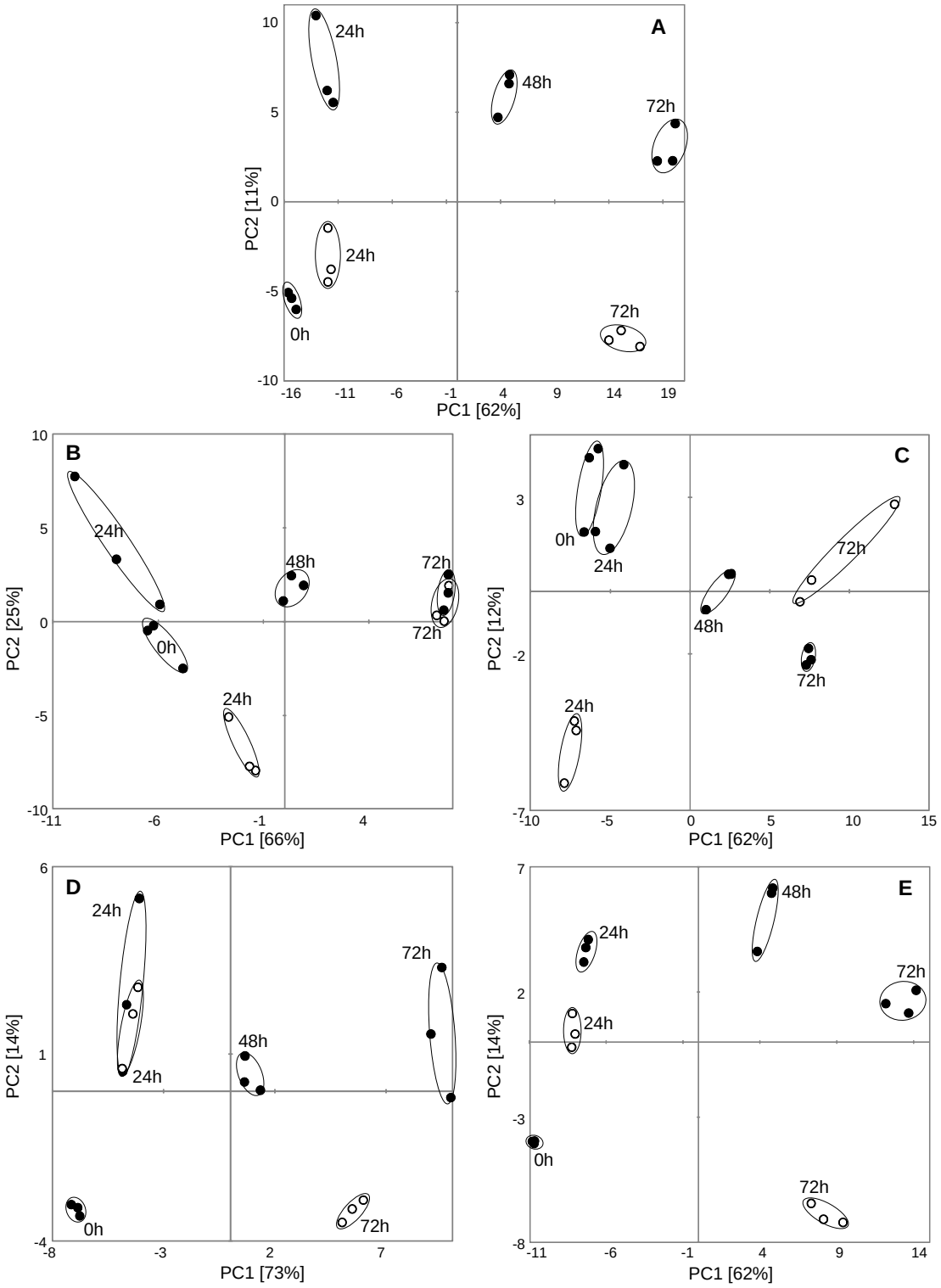


Figure 47: PCA of standardized GC/FID metabolite profiling data of the combined fractions I-IV (A) and the single fractions I (B), II (C), III (D) and IV (E) in the course of industrial-scale sprouting (0, 24, 48, 72h) of mung beans. ● non-washed, O washed with citric acid-containing water.

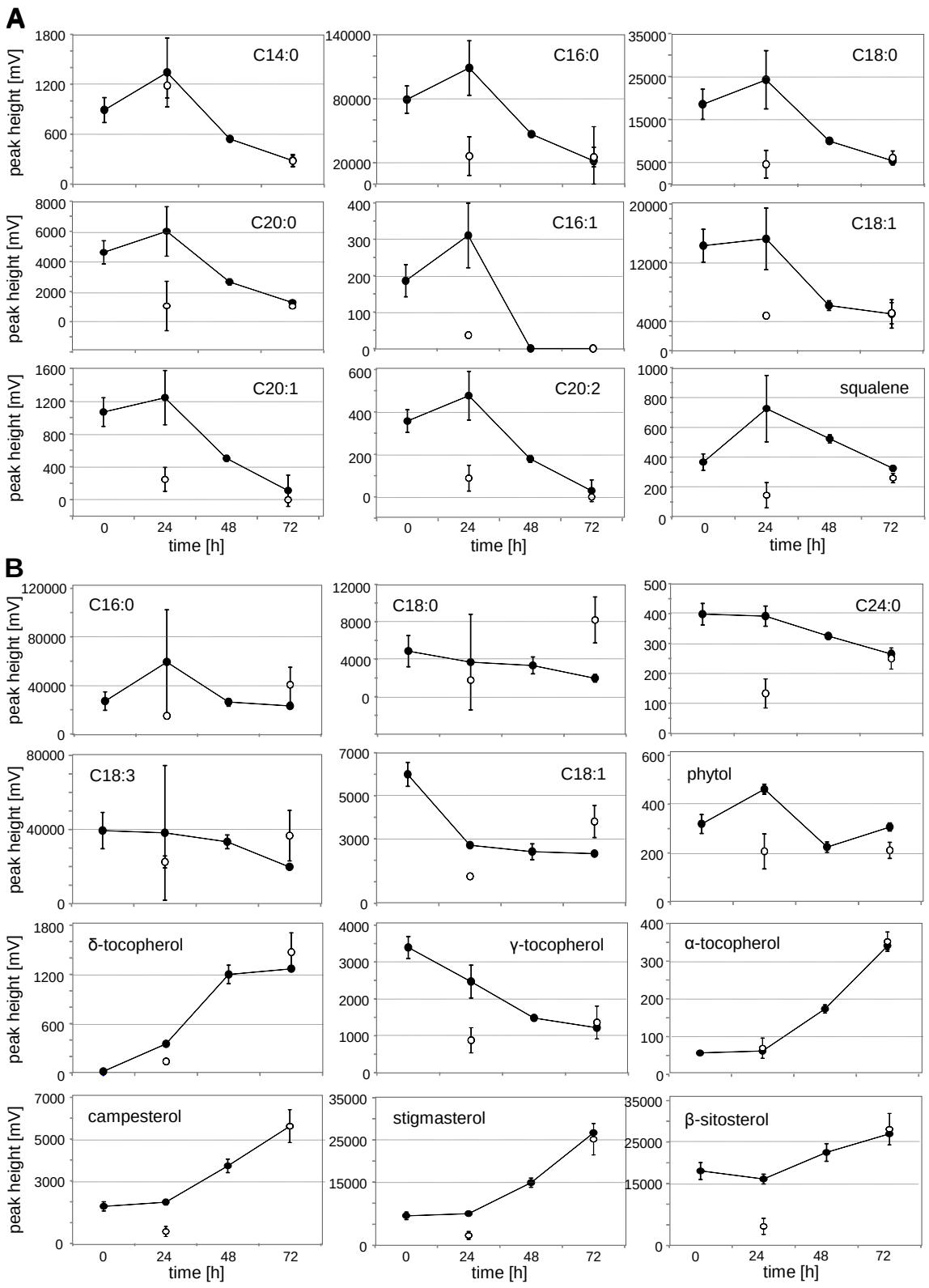


Figure 48: Standardized peak heights for selected compounds determined in fractions I (A) and II (B) in the course industrial sprouting of mung beans obtained from industry. ● non-washed, O washed with citric acid-containing water.

The levels of campesterol, stigmasterol and ß-sitosterol, major sterols in mung beans, were 2 to 4-fold increased compared to the intact seeds. As shown for the major lipids in fraction I, the levels of free fatty acids, tocopherols and sterols in the mini-mung bean sprouts were lower in the treated material (Iw) compared to the non-washed samples. In contrast, the levels of tocopherols and free fatty acids were slightly elevated in the washed sprouts (72h) compared to the non-washed sprouts.

Polar fractions

Compared to non-polar metabolites, the overall changes observed for polar mung bean constituents during germination were more pronounced. Levels of mono- and disaccharides, e.g. fructose, glucose and trehalose were significantly increased in the course of germination, whereas the contents of the tri- and tetrasaccharides raffinose and stachyose were reduced (Figure 49A). The washing of the sprouts (Iw) with citric acid containing tap water resulted in a significant reduction of sugar contents in the sprouts germinated for 72h. For example, the washed sprouts contained 40% less sucrose compared to the non-treated sprouts. This indicates an elution effect for the water-soluble sugars. Considering the dynamic metabolic changes observed in fraction III, this effect was less pronounced for the mini-mung beans (24h).
Similar to the sugars in fraction III, levels of amino acids and organic acids were lower in the treated sprouts (Iw) compared to the non-washed sprouts (In). As an expected result, the citric acid contents in the industrial produced materials (24h, 72h) after washing with citric acid-containing tap water were found to be higher compared to the non-treated materials. A significant loss of GABA was observed after the industrial washing step after 72h (Figure 49B). Levels of the biogenic amines putrescine and ethanolamine increased significantly during the germination period (Figure 49B). On the other hand, compared to the non-washed 72h sprouts (In), the treatment of the industrially produced sprouts (Iw) with citric acid-containing tap water (pH 2.8-2.9) led to a significant reduction especially of the biogenic amine putrescine.

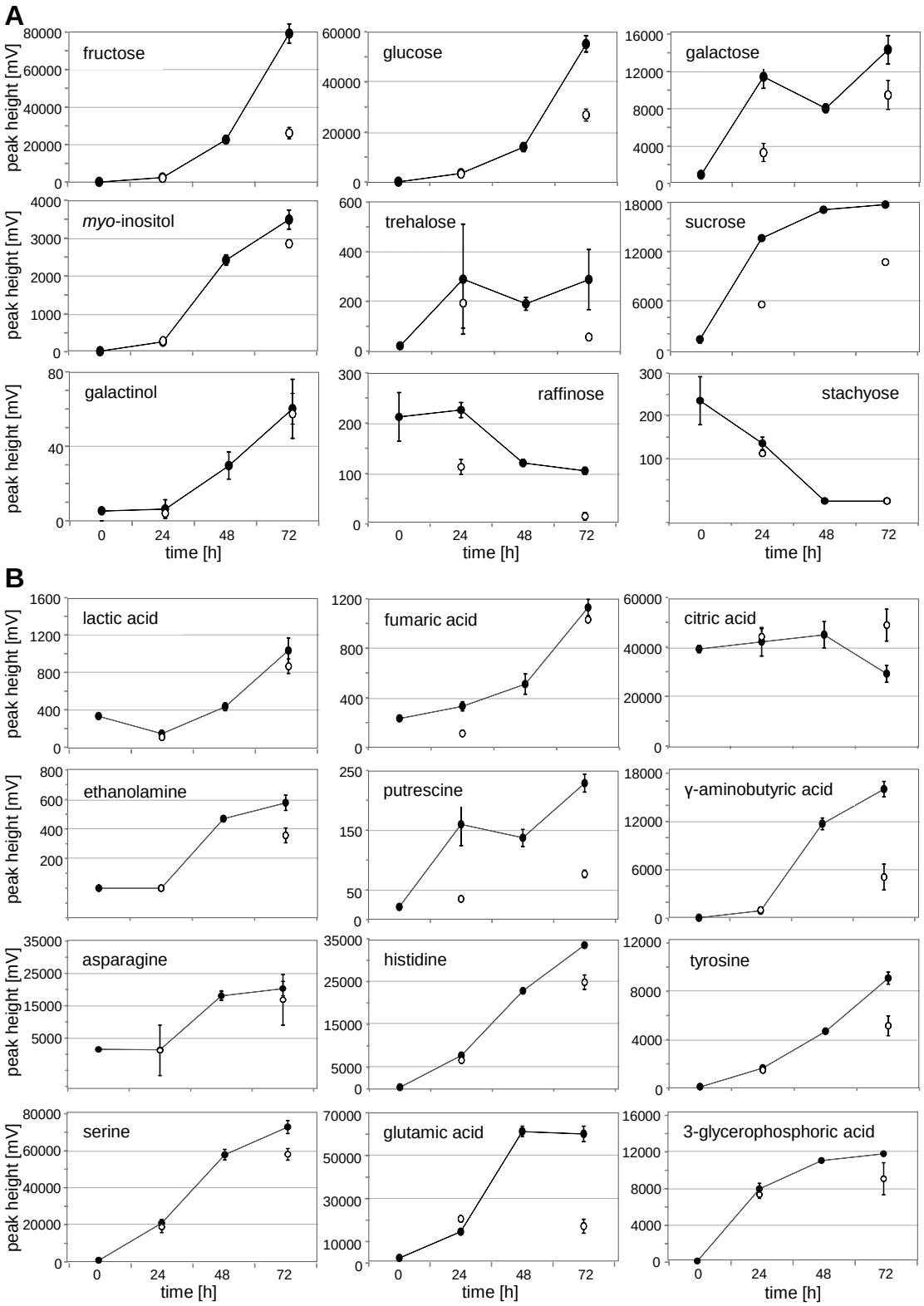


Figure 49: Standardized peak heights for selected compounds determined in fractions III (A) and IV (B) in the course of industrial sprouting of mung beans obtained from industry. ● non-washed, O washed with citric acid-containing water.

4.3.6.3 Conclusions

The washing of intact mung beans with Millipore water and methanol prior to the laboratory-scale sprouting process led to negligible alterations in the metabolite profiles of the final sprouts compared to the non-washed sprouts. The industrially produced sprouts washed with citric acid (Iw) could be clearly distinguished from the non-washed material with regard to both the appearance and metabolite profiles.
In view of the microbial status of sprouted mung beans (Weiss et al., 2007; Mohle-Boetani et al., 2009; Peñas et al., 2010), metabolite profiling can assist in the safety assessment of sprouted foods. By establishing correlations between metabolite profiles of mung bean sprouts and their microflora, valuable indicators helping to predict the sprouting process and the quality of the final food may be elaborated. However, further metabolite profiling studies are required to determine the influence of different treatments on the respective metabolite profiles of the mung bean sprouts. Ideally, a set of biomarkers could be developed representing the course of the sprouting process especially regarding product quality and safety.

4.3.7 Conclusions

Several factors, e.g. incubation temperature and washing treatment, have a considerable impact on the germination process of mung beans. The data obtained demonstrated the suitability of the applied metabolite profiling procedure to assess the tested impact factors on the metabolite profiles of sprouting mung beans. For the factors genotype, temperature and illumination, metabolite profiling revealed differently pronounced influences on the metabolic phenotypes of sprouted mung beans. Although multivariate analysis allowed a clear separation of the two intact mung bean varieties, these differences became less obvious compared to the significant germination time-dependent metabolic changes in the course of sprouting. However, a broad metabolite profiling-based screening of various mung bean genotypes grown at different environmental conditions may help to identify marker metabolites which could be used to predict the germination progress. As expected, higher germination temperatures led to a more rapid sprouting process. On the other hand, it has to be considered that higher incubation temperatures may also potentiate the activity of microorganisms. Therefore, microbiological studies on

sprouting mung beans are necessary to be able to correlate potential spoilage indicators with bacterial counts. Germination of mung beans under the influence of illumination was shown to result in a change of both color and metabolite profiles compared to sprouts produced in the dark. Consumers might prefer sprouts germinated in the dark as their white color apparently implicates a fresher status than sprouts exposed to illumination. From a nutritional point of view, it has to be assessed whether the metabolic differences observed, e.g. the consistently decreased sugar contents in the illuminated mung bean sprouts, are desirable traits.

4.4 STORAGE OF MUNG BEAN SPROUTS

4.4.1 Storage of sprouts at different temperatures

Commercially available raw mung bean sprouts should be consumed preferably at the same day of purchase. Although fresh mung bean sprouts are cooled during distribution and storage in the supermarkets, the shelf life of the sprouts is limited to a few days. In the present study, metabolite profiling was applied to investigate the influence of the storage temperature on the metabolite profiles of mung bean sprouts. Therefore, mung bean sprouts were purchased at a local supermarket at the day of the sprout expiration date specified on the package. The sprouts were stored in a refrigerator (10°C) and at room temperature (24°C), respectively. Samples were taken after 48h and 96h for the sprouts stored at 10°C and after 24h and 48h for the sprouts stored at 24°C. After freeze-drying and milling, the flours were subjected to the metabolite profiling procedure.

For both storage conditions the morphological changes were recorded and compared. Figure 50 illustrates the mung bean sprouts over the storage period at room temperature and at refrigerator temperature. The sampling at each time point revealed significant changes in the shoots. At room temperature, a clear change in morphology and color occurred (Figure 50B). In the fresh state, the shoots were still completely white and the green-yellow cotyledons were visible. After a storage time of 24h, the color of the shoots was dark green to brown. After 48h, a change in the form was recognizable. The shoots and leaves showed a brown color and the scion stem was no longer standing in front, but was contracted and curved. In addition, the consistency of crunchy fresh sprouts has been negatively affected. Sprouts began to become softer and slimy. The observable water efflux indicated an advanced cell wall degradation progress and the slimy surface was suggested to be a result from microbial activity. In contrast to the storage at room temperature, sprouts stored in the refrigerator did not alter their appearance during the first 48h. However, sprouts stored for 96h showed first changes of consistency (Figure 50A).

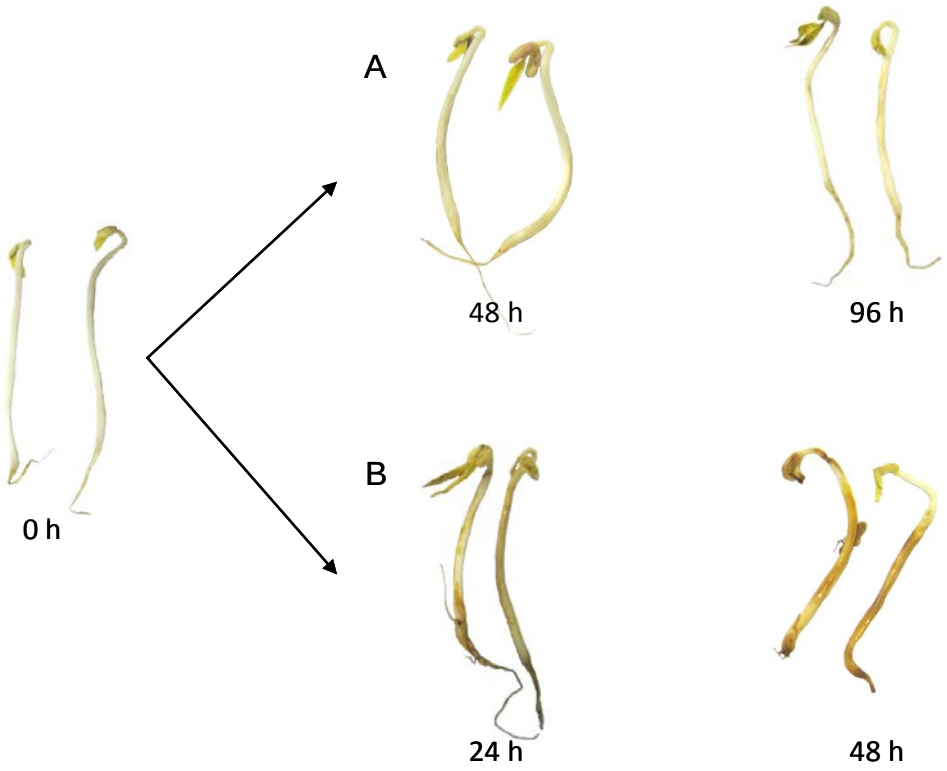


Figure 50: Mung bean sprouts obtained from a local supermarket (0h) stored at 10°C (A) and at 24°C (B).

4.4.2 Multivariate analysis

The sprouts stored at room temperature differed visually significantly from the sprouts stored in the refrigerator. To investigate storage time-dependent metabolic changes, multivariate analysis by means of PCA has been applied. Figure 51 shows the principal component analyses for the data of the combined fractions I-IV from the storage experiments at room temperature and in the refrigerator.

Figures 51A and B reflect the separate metabolite profiling data of sprouts stored at room temperature and in the refrigerator, respectively. Both sprouting conditions are represented by curves in the PCA. Samples from the first time points after 24h and 48h, respectively, were separated on the second PC, whereas stored sprouts representing the final time points were separated on the first PC from the fresh sprouts.

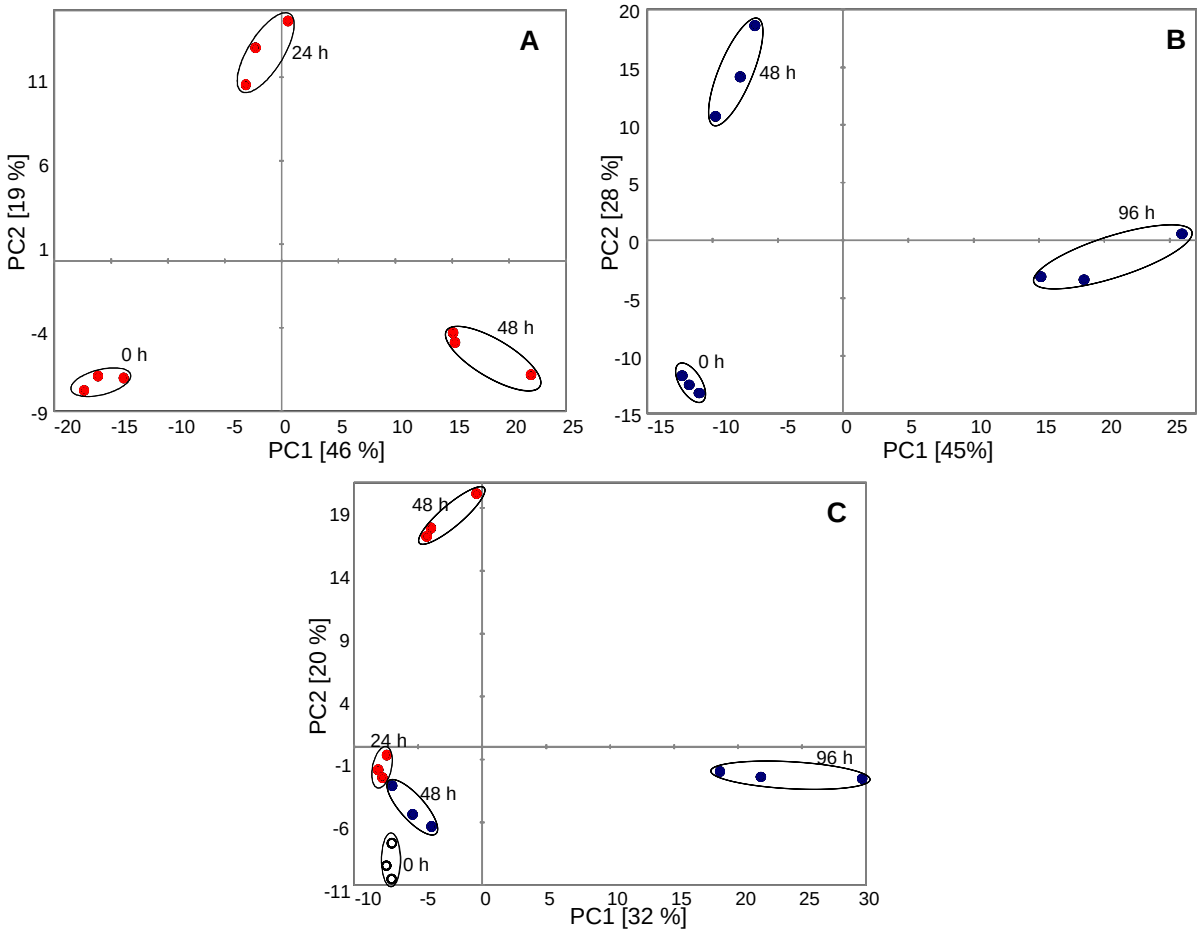


Figure 51: Principal component analysis of standardized GC/FID metabolite profiling data of fractions I-IV from sprouts stored at 24°C (A) and at 10°C (B); the combined PCA for both temperatures is shown in (C).

Based on the PCA containing the metabolite profiling data from both storage conditions, differentiations in the metabolite profiles became obvious (Figure 51C). Compared to the time points after 24h at 24°C and 48h at 10°C, a clear separation of the clusters was observed along both principal components after storage times of 48 hours at room temperature and 96 hours of storage in the refrigerator. At room temperature (24°C), samples cluster along the second principal component (20% of total variation). Sprouts stored at 10°C in the refrigerator cluster along PC1 (32% of total variation) (Figure 51C).

4.4.3 Univariate analysis

The changes in standardized peak heights of selected fatty acid methyl esters, free fatty acids and sterols from fractions I and II, respectively, are shown in Figure 52.

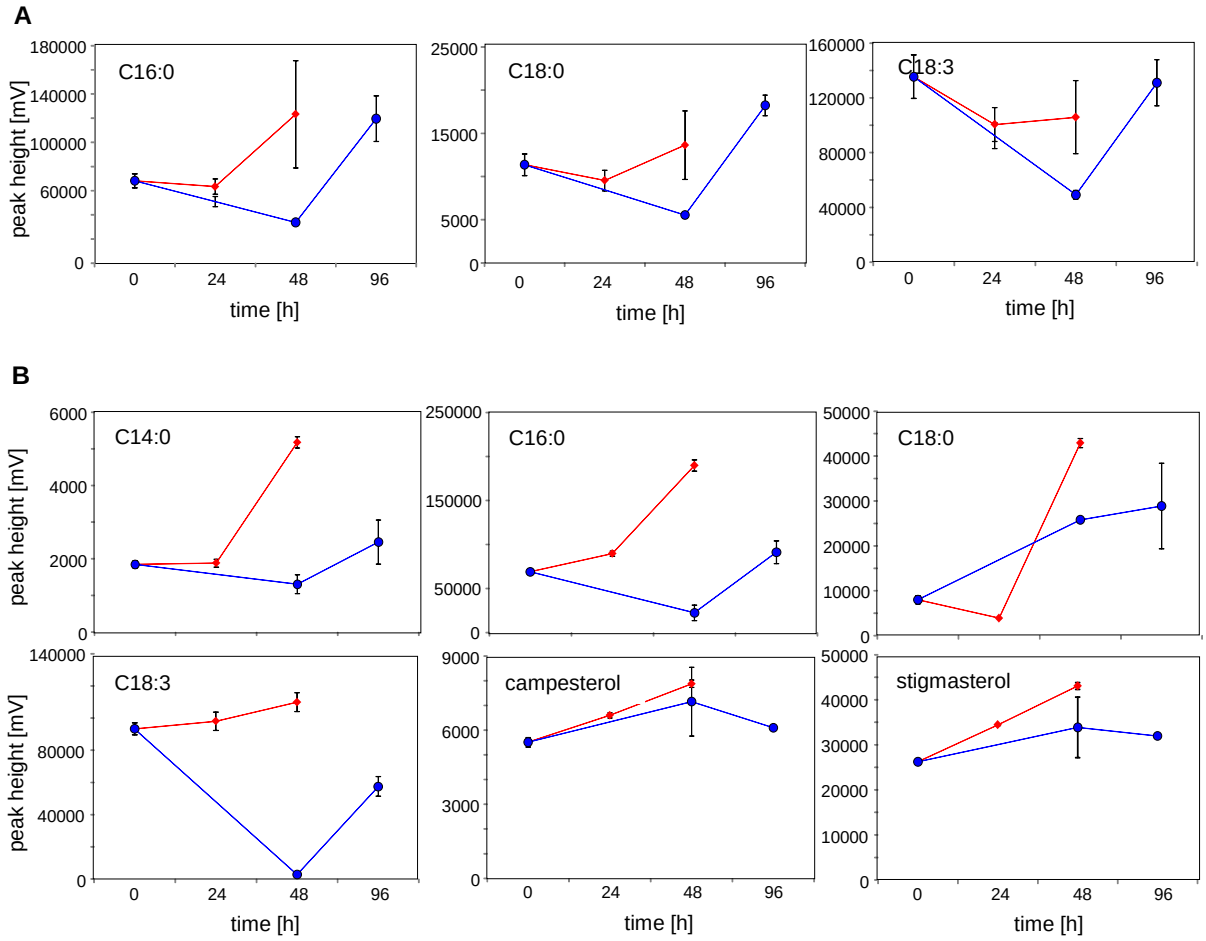


Figure 52: Standardized peak heights for selected compounds determined in fractions I (A) and II (B) in the course of the storage of mung bean sprouts at 10°C (●) and 24°C (●).

Compared to the fresh sprouts, after storage periods of 24h at 24°C and 48h at 10°C, respectively, slightly decreased contents of fatty acid methyl esters were observed (Figure 52A). However, at the latter stages of both storage conditions, increased levels of major lipophilic compounds were determined. Similar to the major lipids, minor lipids apparently increased after storage of 48h and 96h at 24°C and 10°C, respectively (Figure 52B). At the latter stages of storage, the sprouts exhibited liquefaction effects under both temperature conditions. Therefore, the elevated

concentrations of non-polar constituents in the freeze-dried materials of the stored mung bean sprouts may not result from a new formation but from an efflux of water-soluble polar constituents.

The changes in standardized peak heights of selected sugars, acids and amino acids from fractions III and IV, respectively, are shown in Figure 53.

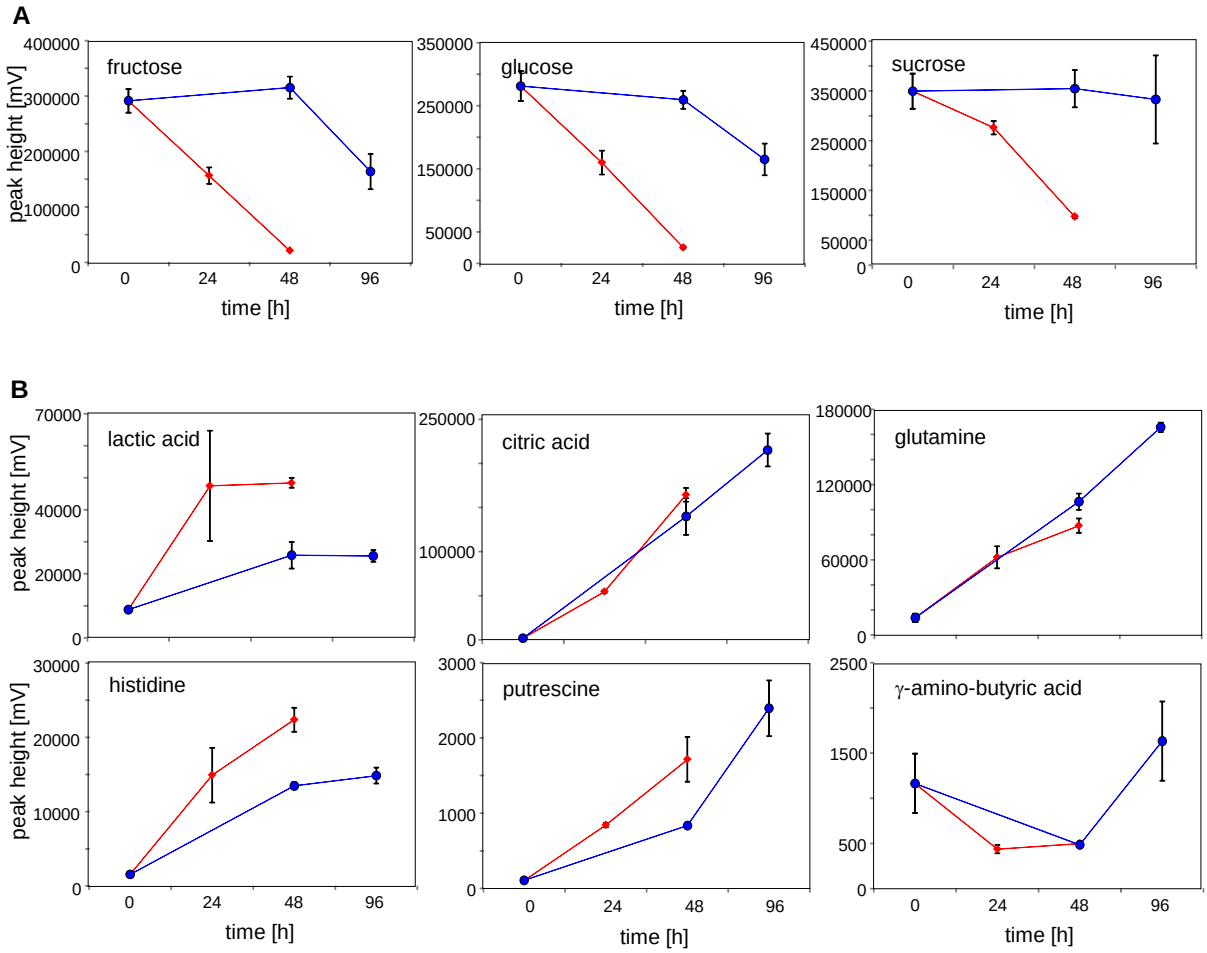


Figure 53: Standardized peak heights for selected compounds determined in fractions III (A) and IV (B) in the course of the storage of mung bean sprouts at 10°C (●) and 24°C (●).

The advanced cell wall degradation progress led to an observable water efflux for the sprouts stored for 48h and 96h at 24°C and 10°C, respectively. This effect may explain the pronounced reductions of the water-soluble sugars in the stored sprouts. In addition, microbial activity might be responsible for the significant reductions in the sugar contents.

The strong increase of citric acid after the second sampling is suggested to be a result from the carbohydrate degradation. Increased disposal of glucose leads to an increased citric acid formation. The rapid increase in lactic acid content after a total of 96h at refrigerated storage may also be attributed to microbial activities.

5 SUMMARY

A metabolite profiling methodology based on capillary gas chromatography was applied to investigate time-dependent metabolic changes in the course of the sprouting of mung beans (*Vigna radiata*). Intact mung beans and sprouting samples taken during the germination process were subjected to an extraction and fractionation procedure covering a broad spectrum of lipophilic (e.g. fatty acid methyl esters, hydrocarbons, fatty alcohols, sterols) and hydrophilic (e.g. sugars, acids, amino acids, amines) low molecular weight constituents. Investigation of the obtained polar and non-polar fractions by GC resulted in the detection of more than 450 distinct peaks of which 146 were identified by means of MS. Statistical assessment by means of multivariate (e.g. principal component analysis) and univariate data analyses demonstrated the suitability of the applied approach to follow the metabolic changes in the course of sprouting processes in a reproducible way. The dynamic changes of single metabolites revealed significantly increased levels of monosaccharides, organic acids and amino acids and a decrease in the levels of triglyceride-derived fatty acids during germination.

Various factors, i.e. genotype and growing environment of intact mung beans, as well as temperature, illumination and washing treatments in the course of the incubation process, were shown to impact the metabolite profiles of sprouting mung beans. Two different mung bean materials were subjected to three independent sprouting procedures under similar conditions to investigate the genetic influence on the metabolite profiles. PCA analysis of the metabolite profiling data revealed reproducible reflections of the three conducted sprouting processes. Thus, the applied methodology allowed consistent differentiations of the two mung bean materials in the course of germinations demonstrating the robustness of the applied methodology. In addition to the factor genotype, the growing-related environmental influence on the mung bean metabolite profiles was assessed by investigating two mung bean genotypes grown at dry and rainy seasons in Thailand. Differences in the metabolite profiles of intact mung beans were observed for the two mung bean materials as well as for the respective growing environments. However, considering the metabolic changes during the sprouting process, the influence of the factor genotype was shown to be more pronounced than the impact of the dry and rainy growing seasons.

In order to test the impact of incubation temperature on the metabolite profiles of sprouting mung beans, one batch of material was germinated at two different temperatures. The results obtained by the sprouting of mung beans at 25°C and 35°C confirm the considerable influence of the incubation temperature on the time-dependent metabolic changes. The mung beans sprouted at the higher temperature exhibited an accelerated metabolism resulting in metabolic changes at a very early stage of the sprouting process. The fast sprouting progress led to an early increase of nutritionally relevant metabolites, such as monosaccharides and free amino acids, whereas the levels of less desired tri- and tetrasaccharides were quickly reduced.

For the investigation of the influence of illumination on the metabolite profiles of sprouting mung beans, samples were germinated under artificial light exposure and comparatively analyzed to mung beans sprouted in the dark. Sprouting of mung beans with and without illumination did not lead to significant differences in the spectrum of low molecular weight components covered by the applied approach.

Metabolite profiling was also applied to investigate the impact of washing steps of mung bean seeds prior and during the sprouting process. Millipore water and methanol were used as washing agents prior to germination at a laboratory scale. Except for some amino acids, no obvious differences between metabolite profiles of the Millipore water and methanol-treated and non-treated mung beans were detected. In addition, the impact of an industrial-scale mung bean sprouting including a washing step with citric acid-containing water was investigated. In contrast to the washing procedures conducted prior to germination, the washing procedure of already sprouted mung beans led to significant changes in the levels of both non-polar and polar metabolites, including biogenic amines as potential indicators for microbial activities.

Finally, metabolite profiling was applied to investigate the metabolite profiles of mung bean sprouts stored beyond the given expiration date. Significant changes in both appearance and metabolite profiles were observed for the sprouts stored at temperatures of 10°C and 24°C; levels of carbohydrates were shown to decrease, whereas contents of acids were shown to increase.

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