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Shewry, P. R., Rakszegi, M., Lovegrove, A., Amos, D., Corol, D. I., Tawfike, A., Miko, P. and Ward, J. L. 2018. Effects of Organic and Conventional Crop Nutrition on Profiles of Polar Metabolites in Grain of Wheat. *Journal of Agricultural and Food Chemistry - JAFc*. 66 (21), pp. 5346-5351.

The publisher's version can be accessed at:

- <https://dx.doi.org/10.1021/acs.jafc.8b01593>

The output can be accessed at: <https://repository.rothamsted.ac.uk/item/847q5/effects-of-organic-and-conventional-crop-nutrition-on-profiles-of-polar-metabolites-in-grain-of-wheat>.

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Effects of Organic and Conventional Crop Nutrition on Profiles of Polar Metabolites in Grain of Wheat

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Supporting Information

ABSTRACT: The profiles of polar metabolites were determined in wholemeal flours of grain from the Broadbalk wheat experiment and from plants grown under organic and low-input systems to study the effects of nutrition on composition. The Broadbalk samples showed increased amino acids, acetate, and choline and decreased fructose and succinate with increasing nitrogen fertilization. Samples receiving farm yard manure had similar grain nitrogen to those receiving 96 kg of N/ha but had higher contents of amino acids, sugars, and organic acids. A comparison of the profiles of grain from organic and low-input systems showed only partial separation, with clear effects of climate and agronomy. However, supervised multivariate analysis showed that the low-input samples had higher contents of many amino acids, raffinose, glucose, organic acids, and choline and lower sucrose, fructose, and glycine. Consequently, although differences between organic and conventional grain occur, these cannot be used to confirm sample identity.

KEYWORDS: *wheat, wholemeal, metabolomics, organic agriculture, low-input agriculture*

INTRODUCTION

Although organic cereals are grown over a small area compared to conventional cereal production (accounting for less than 2% of the total area in the U.K.), they command a premium and, hence, form an important sector of the market. This is because they are considered by some as being healthier (although there is little evidence for this^{1–4}) and to have environmental benefits⁵ compared to conventionally produced crops. Consequently, establishing the authenticity and traceability of organic cereals is important for those trading in, processing, and marketing organic foods and products. This is a significant challenge, because grain composition is known to be affected by genotype, climatic factors, and genotype × environment interactions as well as agronomy, with commercial samples of the same genotypes varying widely in composition.⁶ Because of this, it is unlikely that a simple discrimination between organic and conventionally grown cereals can be made based on single-grain components, and determining multiple components using conventional analytical approaches is too time-consuming and expensive to be used on a routine basis. For example, Stracke et al.⁷ used conventional high-performance liquid chromatography (HPLC)-based systems to show that climatic factors had a much greater impact on the profiles of phenolic acids and carotenoids than the production system.

However, modern metabolomic analyses provide an opportunity to carry out more sophisticated comparisons, by identifying multiple metabolites in a single analytical system and providing data sets that can be compared by multivariate

statistical analysis. For example, Zörb et al.⁸ and Bonte et al.⁹ used gas chromatography–mass spectrometry (GC–MS) to determine polar metabolites in extracts of wholemeal flour made with 80% methanol. Both studies showed that the effects of genotype were greater than those of the farming system (organic or conventional), although the latter study did identify 5 metabolites and 11 unidentified peaks in the chromatograms, which differed significantly between the farming systems.

We have developed high-throughput ¹H nuclear magnetic resonance (NMR) of unpurified extracts made directly into deuterated aqueous methanol as a routine screening tool in plant metabolomics,^{10,11} including grain composition.^{6,12} We have therefore used this approach to explore the relationship between wheat nutrition and grain metabolite composition, first, using samples of grain grown with varying amounts of nitrogen fertilizer, including farm yard manure (FYM), from the Broadbalk long-term continuous wheat experiment at Rothamsted and, second, using grain samples grown in Austria, Switzerland, and the U.K. using organic and low-input conventional systems.^{13–15}

Received: March 28, 2018

Revised: May 1, 2018

Accepted: May 10, 2018

Published: May 10, 2018

MATERIALS AND METHODS

Broadbalk Wheat Samples. Samples of mature wheat grain were obtained from the 2016 harvest from the Broadbalk experiment at Rothamsted Research. This is a long-term (since 1843) field experiment, in which a single cultivar (currently the U.K. winter wheat Crusoe) is grown with various nutrient regimes.¹⁶ Four sections of the experiment were sampled: three continuous wheat sections (0, 1, and 9), one of which had straw incorporation since 1986 (section 0), and one first wheat in an oats–maize–wheat–wheat–wheat rotation (section 4). Within these sections, plots were sampled with zero fertilization, the application of FYM (35 tonnes/ha), and the application of nitrogen (as ammonium nitrate) at 48, 96, 144, 192, and 288 kg/ha. Details of the treatments and grain nitrogen contents are provided in Table S1 of the Supporting Information. Soil inorganic nitrogen was not measured, but a previous study showed 16 and 25 kg of N/ha between 0 and 23 cm and 19 and 20 kg/ha between 23 and 50 cm in plots receiving 144 and 288 kg of N/ha, respectively, and 45 kg of N/ha in a plot receiving FYM. These measurements were made in March with a standing crop of winter wheat but before the application of inorganic fertilizer.¹⁷ Grain samples were ground in a ball mill (Glen Creston, Stanmore, U.K.) to give wholemeal flour.

Low-Input and Organic Samples. Wholemeal samples were obtained from an international research project in which a range of cultivars were grown under “low input” (as defined in Europe, in which high-input systems are the norm) and organic systems in several countries in 2012–2013.^{13–15} The material available for this study comprised 33 cultivars and 3 breeding lines grown under conventionally fertilized low-input conditions in Austria and organic conditions in Switzerland and three cultivars also grown under similar conventionally fertilized low-input and organic conditions in the U.K. (Wakelyns site). In these trials, the low-input plots received 100–120 kg of N/ha, while the nutrients in the organic plots were derived from the previous crops, which were mainly legumes.¹⁵ Samples of 17 wheat cultivars and 2 breeding lines were also obtained from an organic wheat trial carried out at the University of Reading experimental farm (Sonning on Thames) in 2015–2016. Hence, the sets of material varied in geographical origin and year of harvest as well as farming system, providing a good example of the variation that could be expected in commercial samples. Available nitrogen was determined as the sum of nitrite-N, nitrate-N and ammonium-N extracted with potassium chloride (Reading) and as the sum of nitrate-N and ammonium-N extracted with calcium chloride (Switzerland).

Details of the genotypes are given in Table S2 of the Supporting Information, and details of the sites and treatments are given in Table S3 of the Supporting Information.

¹H NMR Spectroscopy. Sample preparation for ¹H NMR spectroscopy was carried out according to the procedures described previously.^{6,12} Wholemeal samples (30 mg) were extracted in triplicate using 80:20 D₂O/CD₃OD containing 0.05% trimethylsilylpropionate-*d*₄ (TSP-*d*₄, 1 mL) as an internal standard. ¹H NMR spectra were acquired under automation at 300 K using an AVANCE spectrometer (Bruker BioSpin, Coventry, U.K.) operating at 600.0528 MHz and equipped with a 5 mm selective inverse probe. Spectra were collected using a water suppression pulse sequence with a 90° pulse and a relaxation delay of 5 s. Each spectrum was acquired using 128 scans of 64 000 data points with a spectral width of 7309.99 Hz. Spectra were automatically Fourier-transformed using an exponential window with a line broadening value of 0.5 Hz. Phasing and baseline correction were carried out within the instrument software. ¹H chemical shifts were referenced to TSP-*d*₄ at δ 0.00.

¹H NMR spectra were automatically reduced, using AMIX (Analysis of MIXtures software, Bruker BioSpin), to ASCII files containing integrated regions or “buckets” of equal width (0.001 ppm). Spectral intensities were scaled to the TSP-*d*₄ region (from δ 0.05 to –0.05). The ASCII file was imported to Microsoft Excel for the addition of sampling/treatment details. Known metabolites were identified via a comparison to a library of standard spectra collected under identical conditions. Individual metabolites were quantified against the known

concentration of the internal standard present in each sample, and data were expressed as milligrams per gram.

Statistical Analysis. Multivariate statistical analysis [principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA)] was conducted on quantified data using SIMCA-P software (version 13, MKS Umetrics). Statistical models were constructed using data scaled to unit variance.

RESULTS

Comparison of Broadbalk Samples. Figure S1 of the Supporting Information shows a typical ¹H NMR spectrum of a polar extract of wholemeal wheat flour. The central part of the spectrum, between about δ 3 and 4.3, comprises overlapping peaks corresponding to the most abundant carbohydrates (sucrose, maltose, raffinose, glucose, and fructose). These peaks are flanked by aliphatic and aromatic regions, corresponding to anomeric protons of sugars, organic acids, amino acids, and other polar low-molecular-mass components, including choline and glycine betaine. The assignments are described in detail by Baker et al.⁶ and Shewry et al.¹² To compare the compositions of grain samples grown under different nitrogen regimes, the major metabolites in the spectra were quantified and compared by multivariate statistical analysis. Figure 1 shows a PCA of the metabolite profiles of polar extracts from the Broadbalk samples, in which principal components (PCs) 1 and 2 account for 40 and 8% of the variation in the data set, respectively. The different N applications are color-coded, while the different symbols represent the four sections (see the Materials and Methods). The nitrogen treatments range from 0 to 288 kg/ha of N as ammonium nitrate, with the “organic” plots receiving 35 tonnes of FYM/ha. The latter contains about 240 kg of N/ha, but only half or less of this appears to be available to the crop.¹⁷ Hence, the nitrogen content of grain from the FYM treatment was most similar to that of the conventionally fertilized grain, which received 96 kg of N/ha (Table S1 of the Supporting Information).

The PCA in Figure 1A shows clear separation of the conventionally fertilized samples in PC2, from the lowest application at the top of the PC and the highest at the bottom, with the FYM samples overlapping with those that received 96 and 144 kg of N/ha. The samples within each treatment show some separation in PC1, but this is not related to the individual sections. However, the most striking separation in PC1 was between the conventionally fertilized and FYM samples, with the latter being toward the left-hand side of the plot.

To identify the compounds responsible for the separations, loadings plots were prepared. Figure 1B therefore shows the compounds that are increased and decreased with high amounts of conventional fertilization (corresponding essentially to PC2). It is not surprising that the major components that are elevated under high nitrogen are amino acids (notably aspartate, asparagine, and tryptophan), together with acetate and choline. In contrast, fructose and succinate are substantially reduced at high nitrogen. A similar loading plot comparing the FYM and conventionally fertilized samples is shown in Figure 1C. In this case, the major differences are increases in a range of amino acids (notably glutamate), sugars (notably sucrose), and organic acids in the FYM plots. However, it is notable that tryptophan, which responds significantly to nitrogen fertilization in the conventionally fertilized samples (Figure 1B), differs little between the conventionally fertilized and FYM samples (Figure 1C).

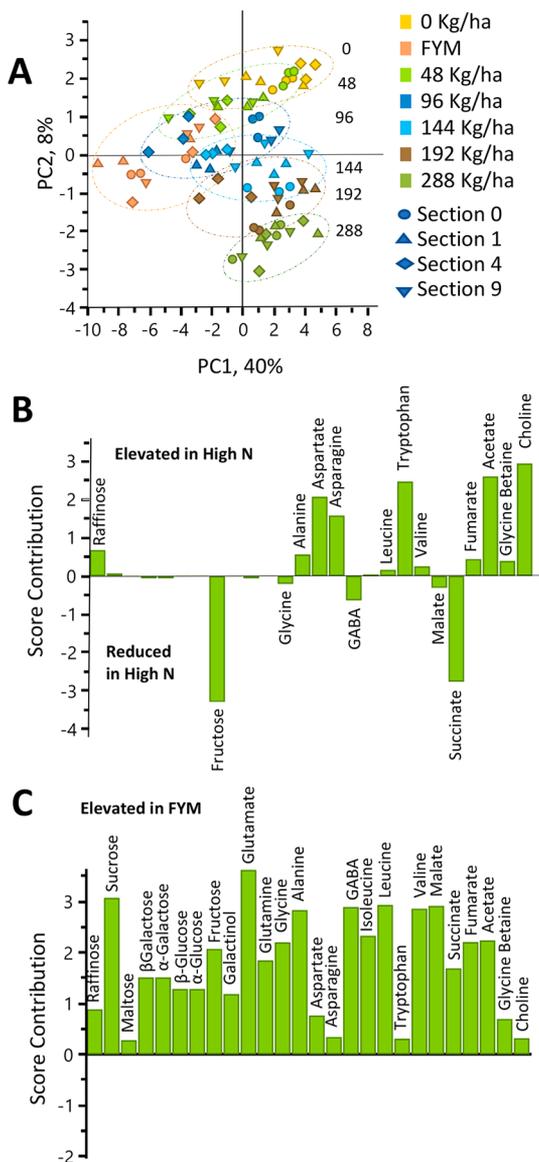


Figure 1. PCA of polar metabolite profiles from the Rothamsted Broadbalk samples: (A) PCA scores plot, colored according to fertilization regime, (B) contribution plot comparing high and low N, and (C) contribution plot comparing FYM and N-fertilized samples.

To focus on the differences between the conventionally fertilized and FYM samples, a supervised multivariate analysis, OPLS-DA, was used to compare only differences relating to the FYM and 96 kg of N/ha treatments (Figure 2). This clearly separated the two sets of samples in the x axis, with both sets varying in the y axis. The contribution plot (Figure 2B) essentially confirmed the analysis in Figure 1C, although in this case, maltose, tryptophan, and glycine betaine are reduced in the FYM samples.

Comparison of Organic and Low-Input Conventionally Grown Samples. Although the Broadbalk experiment provides well-characterized material from plots receiving conventional and organic fertilizer, it is not typical of commercial organic farming, in that the other farming inputs are not organic and the amount of nitrogen applied as FYM is very high compared to commercial systems in which leys, legumes, and green manures are more widely used to provide sources of nitrogen. We therefore compared samples from

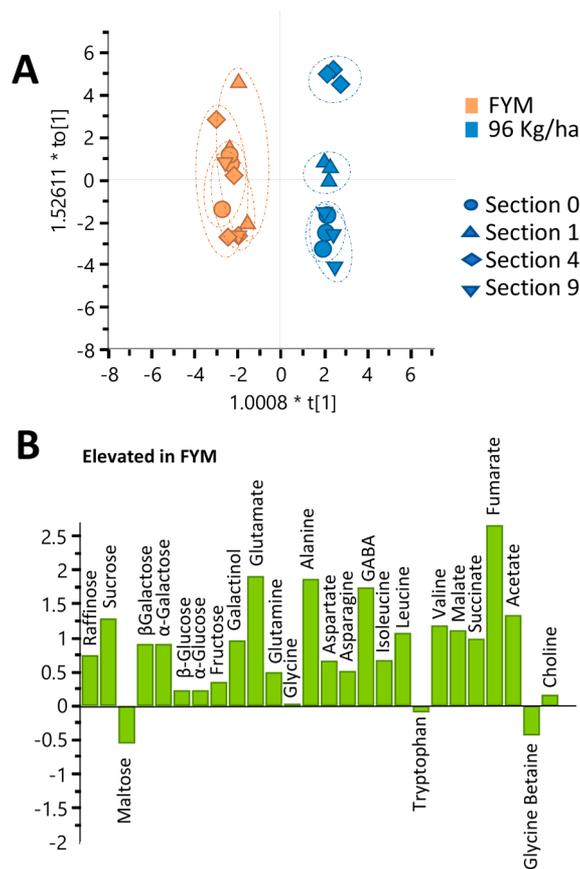


Figure 2. OPLS-DA of polar metabolite profiles of grain samples from the Rothamsted Broadbalk experiment (FYM and 96 kg of N/ha only): (A) scores plot, colored according to the fertilization regime, and (B) contribution plot comparing FYM and 96 kg of N/ha.

organic and conventionally fertilized low-input field experiments, using organic wheat grown in Switzerland (36 genotypes), conventionally fertilized wheat grown in Austria (36 genotypes), organic and conventionally fertilized wheat grown at Wakelyn in the U.K. (3 cultivars), and organic wheat grown on a second U.K. site at Reading (19 genotypes). This material therefore represents a wide range of environments as well as cultivars and farming systems.

Although the mean metabolite concentrations determined for the sample sets (Table S4 of the Supporting Information) showed relatively small differences between treatments, PCA of the NMR profiles (Figure 3A) (with PC1 and PC2 accounting for 35 and 26% of the variation, respectively) showed a clear separation into two groups, with the Reading organic samples being separated from the other sample sets. However, although the Swiss, Austrian, and U.K. organic and low-input samples form a single cluster, some separation is observed in both PCs, and this separation is shown more clearly in Figure 3B, in which only these sample sets are analyzed. This analysis accounts for 85% of the variation in the data set, with PC1 accounting for 73% and PC2 accounting for 12%. The U.K. samples were grown in the same location, with clear separation between those grown under low-input conventional and organic conditions. However, although the Swiss organic samples and the Austrian conventionally fertilized low-input samples are concentrated in the same areas of the PCA separation as the organic and conventionally fertilized low-input samples from

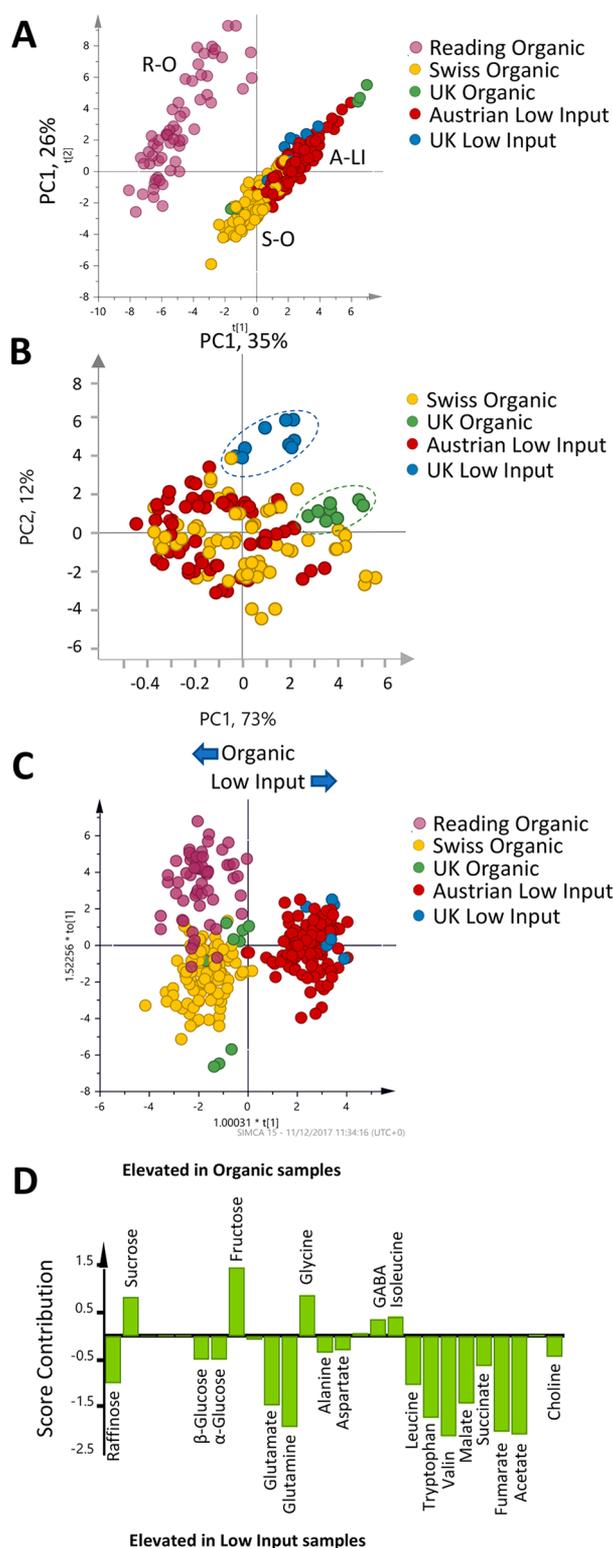


Figure 3. Multivariate analysis of grain from organic and conventionally fertilized field trials in the U.K. (Wakelyns organic and low input and Reading organic), Austria, and Switzerland: (A) PCA scores plot, colored according to fertilization regime and growth location, (B) PCA scores plot of grain grown in the U.K. (Wakelyns only), Austria, and Switzerland, colored by fertilization regime and location, (C) OPLS-DA of grain grown in the U.K., Austria, and Switzerland, colored according to fertilization regime and growth location, and (D) contribution plot comparing organic samples and conventional low-input samples.

the U.K., respectively, they were widely spread, with the two sets of samples overlapping.

Nevertheless, despite the failure of PCA to clearly separate the organic and conventionally fertilized samples, it is still possible to focus on the differences associated with nutrition using supervised multivariate OPLS-DA (Figure 3C). This gives a good separation of the organic and low-input samples, on the left- and right-hand sides of the separation, respectively, and also partial separation of the Reading organic samples from the other organic samples.

The contribution plot (Figure 3D) for the OPLS-DA separation (Figure 3C) shows that, in contrast to the Broadbalk samples shown in Figure 2, the low-input samples all have higher contents of a number of metabolites than the organic samples, including a range of amino acids (including glutamine, glutamate, leucine, tryptophan, and valine), raffinose (a trisaccharide), glucose, organic acids, and choline. However, the organic samples were higher in some metabolites, notably sucrose, fructose, and glycine.

These analyses therefore suggest that the precise farming system (which differed between the Reading organic experiment and other samples) as well as the cultivar, year, type of nutrition, and location affect grain composition. Although the present study was not intended to elucidate these effects, it should be noted that metrological data for the sites show wide variation in temperature and precipitation, including in the final 100 days of growth (which includes grain development and desiccation) (Table S3 of the Supporting Information), and that a previous study showed significant positive and negative correlations between grain components and these factors.¹⁸

DISCUSSION

The Broadbalk FYM plots are not fully organic, and the amount of nitrogen applied is greatly above the amounts that are available to the crop in most organic systems. For example, although no nitrogen was applied to the organic treatments, the available soil nitrogen in the Reading plots was measured as 19.6 kg/ha and in the Swiss plots was measured as 43.1 kg/ha, as compared to 250 kg/ha applied nitrogen (of which over half appears to be available) in the Broadbalk treatment. This contrasts with about 120 kg/ha nitrogen applied in the Austrian and U.K. low-input treatments. Hence, the lower proportions of metabolites, notably amino acids, in the organic samples may be related to the lower amounts of available nitrogen.

Differences relating to total nitrogen application could be avoided when comparing the Broadbalk samples by selecting grain of similar nitrogen content to the FYM samples from a range of application rates. In this case, although the grain protein contents were similar, the grain from the FYM plots contained higher concentrations of a range of polar metabolites, including amino acids. Hence, it is to be concluded that the contents of polar metabolites, including many amino acids, are related to the farming system (presumably the form in which nitrogen is available to the plant) as well as to the total nitrogen availability.

Allowing for these effects, it is also of interest to determine whether nutrition affects the patterns of metabolites that are accumulated as well as their amounts and, in particular, whether it is possible to identify a pattern that is diagnostic for organically grown grain. In comparison of Figures 1C, 2B, and 3D, they show that some components, such as glutamate and glutamine, appear to be elevated in response to total nitrogen. However, two compounds differ from this trend, being only

marginally increased in the Broadbalk FYM samples and clearly lower in the organic samples. These are tryptophan and choline.

Several other studies have also used metabolite profiling to compare grain from conventionally grown and organic wheat and generally showed little difference.^{8,19} Bonte et al.⁹ showed that 5 metabolites differed consistently in concentration between 11 cultivars grown under conventional and organic conditions, including tryptophan, which was consistently lower in the organic samples. However, they also showed lower concentrations of alanine and γ -aminobutyric acid (GABA) in the organically grown grain, whereas these were only slightly lower and slightly higher, respectively, in the organic samples shown in Figure 3, and both were increased in the Broadbalk FYM samples. The study did not report the concentration of choline; thus, the only consistent effect observed in the two studies is the decreased concentration of tryptophan.

Hence, it is concluded that, although conventionally grown and organic wheat grain do exhibit differences in metabolite profiles related to the farming system, these are not consistent between sites and, hence, metabolite composition cannot be used as a diagnostic criterion to identify organic samples.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b01593.

Details of grain samples harvested in 2016 from the Rothamsted Broadbalk experiment (Table S1), details of genotypes, sites, and farming systems of grain samples (Table S2), growing conditions and management practices at organic and low-input fields of the three countries (2012–2013) (Table S3), mean concentrations of polar metabolites (mg/g) in the sets of low-input and organically grown wheat samples (Table S4) and typical 600 MHz ¹H NMR spectrum of wholemeal flour extracted with 20:80 CD₃OD/D₂O (Figure S1) (PDF)

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Funding

Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the U.K. and the work forms part of the Designing Future Wheat Strategic Program (BB/P016855/1). The Broadbalk experiment is part of the Rothamsted Long-Term Experiments National Capability (LTE-NCG) supported by the BBSRC (BBS/E/C/000J0300) and the Lawes Agricultural Trust.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Swiss organic samples were kindly provided by Jürg Hiltbrunner (Agroscope, Zürich, Switzerland), and Austrian low-input samples were kindly provided by Franziska Löschenberger (Saatzucht Donau GmbH & Co. KG,

Probstdorf, Austria). U.K. (Waklyns) samples were developed in the frame of the FP7 Program of the European Community under Grant Agreement 245058-SOLIBAM. U.K. (Reading) samples were developed as part of the FP7 Program of the European Community Wheatalbi under Grant Agreement FP7-613556 and were provided by Caroline Hadley (Crop Research Unit, University of Reading, Reading, U.K.). The authors thank Andy Macdonald and colleagues at Rothamsted Research for providing the Broadbalk samples and Margaret Glendining for providing data in Table S1 from the electronic Rothamsted Archive (e-RA, <http://www.era.rothamsted.ac.uk/>).

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