Studying the effect of storage conditions on the metabolite content of red wine using HILIC LC-MS based metabolomics

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Abstract

The main aim of this work was to develop an untargeted normal phase LC-MS method, starting from a targeted method already validated for the analysis of 135 polar metabolites. Since the LC instrument and column were the same, most of the chromatographic conditions remained identical, while the adaptations focused on maintaining the ionic strength of the eluents constant. The sample preparation was simplified and the effectiveness of LC-MS for long batches was evaluated, in order to record the maximum number of metabolites with good chromatographic resolution and the best MS stability and accuracy. The method was applied to study the influence of storage conditions on wine composition. Slightly sub-optimum storage conditions had a major impact on the polar metabolite fingerprint of the red wines analysed and the markers revealed included phenolics, vitamins and metabolites indentified in wine for the first time (4-amino-heptanedioic acid and its ethyl ester).

Keywords: holistic, wine metabolomics; wine conservation; method development; primary metabolites; metabolomics; normal phase LC; QTof MS; HILIC, *Vitis vinifera*.

1. Introduction

Metabolomics, or metabolic fingerprinting, is used for comprehensive characterisation of molecules in the biological system investigated. In contrast to targeted methods, where the analyte(s) of interest is well known and the scope very specific, in untargeted metabolomics the aim is to achieve the widest possible metabolic coverage in an unsupervised manner. The idea has found application in life, environmental, food and other sciences (Baker, 2011; Gika, Theodoridis, Plumb, & Wilson, 2014; Gika, Wilson, & Theodoridis, 2014; Nicholson & Lindon, 2008). Although the technique has been known since the 1960s (Dalgliesh, Horning, Horning, Knox, & Yarger, 1966), major evolution started in around 2000 and since then the number of publications and active researchers in the field of metabolomics has increased rapidly (Gika, Wilson, et al., 2014). Because of the high cost and specific expertise in different fields needed, the first publications in food science appeared later (Cubero-Leon, Peñalver, & Maquet, 2014; Oms-Oliu, Odriozola-Serrano, & Martín-Belloso, 2013). Oenology and viticulture were among the first sectors in food science to embark on metabolomics studies (Arapitsas et al., 2012; Arapitsas, Speri, Angeli, Perenzoni, & Mattivi, 2014; Castro, Martins, Teixeira, & Silva Ferreira, 2014; Gougeon et al., 2009; Theodoridis et al., 2011).

Since targeted methods have been widely used and taught in universities courses of analytical chemistry for many decades, their applications to food chemistry, biology or medicine are very advanced and the knowledge and expertise for method development is well established in the corresponding industries and regulatory bodies. Untargeted techniques are relatively new, and so there is still debate about experimental design and method validation (among other things). The development of a valid holistic LC-MS method is one of the most critical steps in a metabolomics project, because not only the detection of as many metabolites as possible is required, but also high

instrumental stability and repeatability during analysis, in order to facilitate chromatographic alignment, the accurate definition of metabolic space and marker discovery with as few false negatives/positives as possible. Researchers are currently developing their methods based on the experience obtained in the targeted analysis field. Most efforts are directed at 1) improving metabolite detection and identification, mainly taking advantage of recent developments in mass spectrometry technology (Dunn & Hankemeier, 2013; Holčapek, Jirásko, & Lísa, 2012); and 2) data analysis, to look through massive datasets trying to unravel biochemical phenomena and identify trends in complex data. Metabolomics datasets are massive (especially those generated by LC-MS) and can be an attractive asset for chemometricians, programmers, and statisticians (Dunn & Hankemeier, 2013; Gika, Wilson, et al., 2014). This is expected to grow further with the increased availability of data (and metadata) open repositories (Franceschi et al., 2014). Finally the sample preparation methods are in most cases as simple as possible, to avoid the introduction of bias due to the different extraction efficiency observed for different metabolites for example (Mattivi et al., 2012; Noack & Wiechert, 2014; Theodoridis et al., 2011; Wu, Southam, Hines, & Viant, 2008).

Hydrophilic interaction chromatography (HILIC) has recently appeared to be the best LC approach for complementing reverse phase (RP) chromatography in global (untargeted) metabolomics fingerprinting. Because of its different selectivity, HILIC offers better retention for polar metabolites and due to its mechanism it provides better sensitivity (Gika, Theodoridis, et al., 2014; Gika, Theodoridis, Vrhovsek, & Mattivi, 2012; Spagou et al., 2011; T'kindt, Storme, Deforce, & Van Bocxlaer, 2008; Theodoridis et al., 2011). We can divide the published applications of HILIC into two main categories: a) targeted methods focusing on one group of metabolites (Valls, Millán, Martí, Borràs, & Arola, 2009; Vilhena et al., 2014; Willemse, Stander, & de Villiers, 2013; Zhu et al., 2014) and b) untargeted methods which follow a holistic approach (Spagou et al., 2011;

T'kindt et al., 2008; Theodoridis et al., 2011). Our group (Gika et al., 2012) recently developed a targeted HILIC-MS/MS method which covers a wide set (135) of primary and secondary polar metabolites belonging to various molecular classes such as sugars, sugar alcohols, organic acids, bio-amines, amino acids, etc, which play an important role in food and plant chemistry.

Wine can be stored long time and in contrast to most food, ageing is positively correlated with quality. Cheap wines are usually consumed within a short time, while premium quality wines are expected to last and even improve with age, and are frequently stored for up to several years. However, both products should be stored in ideal conditions to allow consumers to experience equal or better quality as compared to the legal requirements and winemakers' standards when the wine leaves the winery. From the winery to the consumer many factors can influence wine quality and nutritional value, especially storage conditions and duration. Optimum storage conditions reduce the risk of deterioration in wine quality, gain customers' confidence and sharpen the competitive edge in the market. Humidity, temperature, light and packaging have the most direct impact on wine quality and nutritional value, so there are various studies in the literature dealing with metabolic changes caused in wine by incorrect and sub-optimal conservation conditions (Arapitsas et al., 2014; Ghidossi et al., 2012; González Marco & Ancín Azpilicueta, 2006; Hernanz et al., 2009; Hopfer, Ebeler, & Heymann, 2012; Loscos, Hernández-Orte, Cacho, & Ferreira, 2010; Monagas, Bartolomé, & Gómez-Cordovés, 2005; Wirth et al., 2010).

First aim of this work was to adapt the above described targeted method and to optimise it to a holistic LC-MS metabolic profiling method. The final goal was to apply the optimized metabolic profiling method to the study of a common oenological problem,

115 namely the effect of typical domestic storage conditions as compared to optimum cellar
116 conditions for five red wines and for a period of 24 months.
117
118 **2. Material and Methods**
119 *2.1. Chemicals*
120 All chemicals used in this study were of the highest purity grade available and
121 purchased from FLUKA Sigma-Aldrich, unless otherwise stated. Water purified with a
122 Milli-Q Water Purification System was used for chromatography and preparation of
123 standard solutions.
124 *2.2. Wines*
125 During 2009 five *Vitis vinifera* L. cv. Sangiovese grape samples from different
126 areas of Montalcino (Tuscany - Italy) were collected, in order to cover the whole
127 production area of Brunello di Montalcino. From each selected vineyard, a total of c. 80
128 kg of grapes was sampled in 20 kg boxes and sent via refrigerated transport (4 °C) to
129 the experimental winery at the Edmund Mach Foundation (Trentino - Italy) on the same
130 day. The grapes from each zone were vinified separately and after malolactic
131 fermentation (March 2010) the young wine samples were filtered and bottled in 375 mL
132 dark glass bottles (~20 bottles for each wine). Further details and basic oenological
133 parameters and analysis can be found in Arapitsas et al. (2014).
134 *2.3. Storage*
135 After bottling, which was considered as time zero in the experimental design, two
136 bottles from each wine was stored at 4 ^{o}C (time zero or reference samples). The rest of
137 the bottles of each wine were divided into two groups and stored in two different
138 conditions: (i) One half was stored in such a way as to mimic typical home/domestic
139 wine conservation, with variable conditions during the year, with medium temperature
140 around 20-22 ^{o}C (only few summer days temperature arrived at 26-27 ^{o}C), in a room

with no light but without temperature or humidity control (domestic) and (ii) the other set was stored in a cellar where the temperature (15-17 ^{o}C) and the humidity (~70%) were controlled, stable and optimum for red wine conservation (cellar). For both conditions, wine bottles were stored in horizontal position inside typical cardboard wine boxes. Every 6 months two bottles of each wine (two from the domestic and two from the optimum conservation conditions) were sampled and brought to 4 ^{o}C. The final storage time was 24 months after time zero, which was the bottling date. All samples were analysed together at the end of the experiment.

2.4. Sample preparation

On termination of the experiment the samples were kept for two months at 4 ^{o}C until analysis. Wines were uncorked under nitrogen atmosphere and an aliquot was transferred into a 15 mL amber vial (filled to capacity). Then, again under nitrogen atmosphere, a quality control (QC) pooled sample was prepared by combining 0.5 mL of each sample.

For the dilution test, the QC sample was diluted 1:1, 1:2, 1:4, 1:6 and 1:9 v/v with acetonitrile (ACN). In the finally adapted protocol, 1 mL of each wine sample was diluted with 2 mL ACN (1:2 dilution) and then 40 µL of the internal standard mix was added. Finally 2 mL from each sample/QC was filtered through 0.2 µm PTFE filters into a 2 mL autosampler amber vial (MS certified) for LC/MS analysis. The internal standard mix contained 14 mg of xanthosine and 25 mg of nicotinic acid in 10 mL of MeOH:H_2O (1:1). For blank samples the same procedure was followed but instead of wine, 1 mL of Milli-Q water was used.

2.5. UPLC-QTOF MS analysis

A Waters Acquity UPLC coupled via an electrospray ionization (ESI) interface to a Synapt HDMS QTOF MS (Waters, Manchester, UK) operating in W-mode and controlled by MassLynx 4.1 was used. All samples were analysed with a 2.1 mm × 150

mm ACQUITY UPLC 1.7 µm BEH amide column (equipped with an ACQUITY UPLC BEH Amide 1.7 µm VanGuard Pre-column) which was maintained at 60 °C and eluted with a multistep gradient over the course of 30 min at 400 µL/min. The gradient started with a 4 min isocratic step at 100% A (acetonitrile–water, 95–5 (v/v), 20 mM ammonium formate), then rising to 28% B (acetonitrile–water, 2–98 (v/v), 20 mM ammonium formate) over the next 21 min and finally to 60% B over 5 min. Then the flow rate was decreased to 250 µL/min in an isocratic step for 0.5 min; after that B was increased to 80% and was kept isocratic for 2 min and finally the column was returned to the initial conditions for a 12 min equilibration. Two cycles of weak and strong solvent washing of the injecting system were carried out between injections. The injection volume was 10 µL and the samples were kept at 4 ºC throughout the analysis. Mass spectrometric data were collected in positive ESI mode over a mass range of 30 to 1000 amu, with scan duration of 0.3 s in centroid mode. The transfer collision energy and trap collision energy were set at 6 V and 4 V. The source parameters were set as follows: capillary 3 kV, sampling cone 25 V, extraction cone 3V, source temperature 150 ºC, desolvation temperature 500 ºC, desolvation gas flow 1000 L/h and nebulizer gas 50 L/h. External calibration of the instrument was performed at the beginning of each batch of analysis. LockMass calibration was applied using a solution of leucine enkephaline (0.5 mg/L, *m/z* 556.2771 for positive ion mode) at 0.1 mL/min.

2.5.1. Dilution test

In order to find the optimum sample dilution 20 injections of each QC dilution were performed, starting with the most diluted sample. Between each dilution set, a blank sample was injected.

2.5.2. MS QTof stability test

Before running the real samples and in order to evaluate LC-MS stability in practical conditions, 50 injections of the QC samples were analysed, with one blank sample injection between every 10 QC injections.

2.5.3. Sample set

The sample set was made up of 5 biological replicates/time points/conditions; specifically, 5 bottles for time zero (reference), 5+5 bottles for the 6 months time point, 5+5 bottles for 12 months, 5+5 bottles for 18 months, and 5+5 bottles for 24 months (5 *domestic* and 5 *cellar*). So in total the sample set included 45 bottles of wine, the QC and the blank. The in-batch order of all samples analysed was randomised by using the http://www.random.org/sequences/ site. At the beginning of each batch one blank and four QCs were injected. After this system equilibration, one QC was injected every six samples and at the end of the batch one blank was injected.

2.6. Data analysis

Raw data were converted into CDF format (Waters Databridge) and then processed using XCMS for feature extraction, grouping and alignment. For peak picking, the centWave algorithm was used with a peak width of 20-80 s and the signal to noise parameter was set to S/N = 4. Data extraction parameters were selected on the basis of the characteristics of chromatographic and mass spectrometric performance. The peak table output of XCMS was then used for statistical analysis with the software EZInfo SIMCA-P version 12.0.0 (Umetrics, Umea, Sweden), using Pareto normalization. The PCA plots of EZInfo SIMCA-P were used for quality control of the data sets by checking the distribution of the QC injections. Orthogonal partial least-squares discriminant analysis (OPLS-DA) was used to find features/tentative markers contributing to group/condition discrimination, based on the t-test with the Mann-Whitney U-test and the S-plot of SIMCA-P software.

Metabolite annotation was performed manually by applying the minimum reporting criteria of the four identification levels reported by Dunn et al. (Dunn et al., 2012). For the 1st annotation level, mass accuracy (with less than 5 ppm error), isotopic distribution, at least one fragment and retention time were used and compared to those of the authentic standard. For the 2nd annotation level, mass accuracy (with less than 5 ppm error), isotopic distribution, at least one fragment, literature information about wine metabolome and chromatographic properties, and external databases such as HMDB, Kegg and MassBank were used. For further annotation exploration also MSe, MS/MS and CAMERA were used. For the 3rd annotation level, where only a chemical formula was given, only mass accuracy (with less than 5 ppm error) and isotopic distribution were used.

Peak integration of all annotated compounds, internal standards and tentative markers was performed using the TargetLynx tool of MassLynx Software. Peaks areas emanating from this data were also used for quality control of the analysis by evaluating the variability of specific metabolites. For this part the raw LC-MS files were used and not the peak table exported from XCMS analysis.

3. Results and Discussion

3.1. Method development

In analytical chemistry method development is an important process, with which researchers try to determine the minimal combination of analytical steps required for the identification and quantification of one or more analytes in a specific matrix. In targeted methods these steps are well defined, and more or less easy to follow and accomplish, since they are based on commercial standards and the final scope is clear and solid. The opposite occurs with untargeted methods, because if the researcher focuses on

242 specific analyte(s) a bias towards that class of analytes is introduced, and the holistic
243 character of the technique is annulled.

244 The main scope of this project was to study the effect of different storage
245 conditions on the metabolic content of red wine products. A method previously
246 developed by our group (Gika et al., 2012), able to quantify up to 135 polar metabolites,
247 covering a central part of the chemical and biological variability of the primary
248 metabolites, with 37 mono-, poly-saccharides and reduced monosaccharides (polyols),
249 37 organic aids, 31 amino acids, 22 amines and other biomolecules such as nucleosides
250 and vitamins was adapted to an untargeted metabolite profiling method. The
251 chromatographic apparatus (Waters Acquity UPLC) and the ESI source of both systems,
252 UPLC-MS/MS and UPLC-QTOF MS, were almost identical, so chromatography and
253 ionization were expected to be similar.

254 As it could be anticipated, because of the different nature of the two approaches
255 some parameters needed to be adapted and optimized in order to transfer the targeted
256 method from triple quadrupole MS to a QTOF-MS untargeted method. The optimized
257 parameters could be divided into two broad categories: sample preparation and LC-MS
258 set up. The construction of this untargeted method started from optimization of LC-MS
259 set-up, because this is more delicate as compared to sample preparation. A robust LC-
260 MS method is a key feature in metabolomics for a number of reasons, especially for
261 correct peak alignment. Poor chromatographic repeatability and peak shape in
262 untargeted data-sets can prove very difficult to treat: peak alignment may fail, thus
263 further complicating bioinformatic analysis; such problems may increase false positive
264 and false negative results. A common practice to avoid misalignment in untargeted
265 metabolomics is to analyse all samples in a single batch with as few interruptions as
266 possible. It therefore becomes priority to keep chromatographic system robustness in

terms of retention time for long periods, covering at least the whole duration of a batch of the experiment.

To have unvarying retention times in HILIC mode it is very important to preserve constant ionic strength during the measurements and throughout the different runs or analytical batches. For this reason, it was decided to use a stock buffer solution for the preparation of both A and B eluent, which is a practice used - but not common - in targeted analysis. A stock buffer solution offers the chance to prepare and re-prepare eluents with the same ionic strength in a convenient, fast and robust way for the whole length of the metabolomic experiment, which may last from a few days to several weeks. The previous buffer concentrations were optimized based on the shape of the peaks detected. It was observed that for some peaks shape was improved by increasing the buffer concentration from 10 to 20 mM. Given that the highest buffer concentration to dissolve in the eluent A was 20 mM (Gika et al., 2012), it was decided to keep both strong and weak eluent at a 20 mM concentration. Both solvents were prepared by using the same stock solution of ammonium formate (4M). For better control of the ionic strength of the eluent, pH was adjusted to 4.8 before final dilution with the organic solvent (+2% ACN for A and +95% ACN for B eluent). In HILIC mode and in contrast to reversed phase LC, analytes often exhibit strongest retention in their ionized form. This tendency copes well with ESI-MS, since analytes elute from the column already ionized.

In HILIC mode if the analytes of interest are acids it is better to work at high to medium pH (2 pH units above analyte pKa values), while for bases the opposite is suggested: low to medium pH (2 pH units below analyte pKa values). Focusing only on one group of metabolites (acids or bases) cancels the holistic character of an untargeted method. Furthermore, the column manufacturer (Waters) advises using intermediate pH values (5-7) for methods targeting various type of analytes. For this reason and after

292 also considering the pKa of ammonium formate, it was decided to adjust the pH of both
293 eluents to 4.8.

294 The next step to consider was sample preparation. For the targeted method three
295 different sample dilutions were used for compound quantification, which is logical if one
296 considers the large number of metabolites and the wide concentration range in real
297 samples (Gika et al., 2012). Such a strategy is not an option in untargeted methods and
298 only one method for sample preparation is used. However, sample preparation should
299 be optimized with two key end-points: 1) to provide the maximum possible number of
300 metabolites and 2) to protect the analytical system to maintain a stable signal throughout
301 analysis. Given that it is not possible to measure the actual number of metabolites
302 existing in the sample, the efficiency of sample preparation is commonly evaluated by
303 the number of features. A very simple definition of a feature is “a molecular entity with a
304 unique *m/z* and retention time”. From our experience (Arapitsas et al., 2012, 2014;
305 Mattivi et al., 2012) in wine metabolomics simple sample preparation can provide
306 chromatograms with fewer features in comparison to typical sample preparation for
307 targeted analysis of wine (e.g. via solid phase extraction, SPE). However, the final result
308 of sample preparation is not biased by definition and from the beginning in favour of
309 certain molecules. In this project, in agreement with previous studies (Arapitsas et al.,
310 2012, 2014) we chose to use only dilution, followed by filtration prior to injection into LC-
311 MS. This compromise was aimed to define the smallest experiment needed to build an
312 holistic view of the polar metabolome.

313 In order to select the optimum dilution factor, four parameters were evaluated:
314 the number of features, the precision of the profile acquired over 20 injections (Figure
315 1A), the grouping shown by the PCA plot (not shown) and the quality of a few selected
316 peaks by visual inspection (Figure 2). A pooled QC sample of all wine samples was
317 prepared and diluted with ACN 1:1, 1:2, 1:4, 1:6 and 1:9 v/v. Then 20 consecutive

318 injections of the undiluted wine and each of the diluted samples were performed with the
319 optimised and adapted LC-MS method. The results of this experiment, shown in Figure
320 1A, illustrated that undiluted wine and the two highest dilutions (1:6 and 1:9 v/v) had a
321 very similar low number of features (c. 6000); but this number for undiluted wine, as
322 expected from our previous experience (Arapitsas et al., 2014), already decreased
323 considerably after 10 injections. The 1:1 and 1:2 dilutions had the highest number of
324 features (between 7000 and 7500), with the first showing a slightly higher number of
325 features but the second showing better stability over the 20 injections. As explained
326 above, system stability was given higher priority in this study and so the 1:2 (wine:ACN
327 v/v) dilution was selected.

328 From the comparison of specific compounds/peaks it was observed that peak
329 resolution improved at higher dilution factors, probably due to the peak broadening
330 observed with the increase in water content in the injection solvent (Figure 2). Wine
331 contains around 85% water and our chromatography starts with 98% ACN. In reversed
332 phase inappropriate choice of injection solvent may result in peak broadening mainly in
333 the first part of the chromatography, but in this HILIC experiment broad peaks and
334 problems caused by inappropriate injection solvent were observed over the whole length
335 of the chromatogram. This was despite the fact that the injection volume (10 µL) was not
336 high for the flow rate used (400 µL/min). Some examples are presented in Figure 2,
337 where it is clear that with the increase in ACN content (from the top to the bottom in the
338 Figure) the peak shape improved, while in the case of sugar alcohols, resolution also
339 increased. The peak shape got sharper not only for the early eluting uridine, but also for
340 proline, which elutes in the middle of the chromatogram, and the late eluting raffinose
341 (Figure 2).

342 In the injections of undiluted wine various double peaks were observed for the
343 same metabolite (as shown for uridine and proline in Figure 2). This phenomenon

344 actually generated a higher number of features. Lower peak resolution (broader peaks)
345 was also observed, which may result in a loss of features for closely-eluting analytes.
346 Finally, detector signal saturation can cause problems such as ion/signal suppression
347 but also problems in terms of mass resolution. The column manufacturer suggests a
348 minimum of 75% ACN in the injection solvent. In this study, the best results were found
349 with 66% ACN (resulting from the dilution of 1:2 v/v wine: ACN), but wine already
350 contains 13% ethanol, which is protic/strong for HILIC solvent. To avoid precipitation
351 problems, sample filtration was performed after dilution, just before LC-MS analysis.
352 The next step was to check system stability/robustness in a batch with a higher
353 number of injections, which should be similar to real experiments and could also function
354 as a pilot study. After a sequence of 55 injections (50 QC injections with one blank
355 injection every 10 QC injections) the system continued to have acceptable signal
356 stability, both in terms of the number of features and the peak area of selected
357 compounds (Figure 1B). This experiment also shows that injection of blanks during an
358 untargeted metabolomics sequence should be carefully planned. As shown in Figure 1B,
359 the number of features for the 21st QC injected following a blank injection is much lower
360 as compared to all other QC injections. This could be attributed to chromatographic
361 instability. At the same time it is possible to note a slight perturbation between the
362 groups of QCs divided by the blanks (Figure 1B). In our experience, this phenomenon is
363 regular rather than an exception. If the injection of blanks during a sequence is
364 mandatory, then it is good practice to include 1 or 2 QC injections before the next real
365 sample injection. Moreover, such pilot studies can provide information about the stability
366 of prepared samples remaining in the autosampler, since the QC is a pooled sample of
367 all samples and should have the same metabolic space and physicochemical properties.
368 The results of this study confirmed the excellent stability of the LC-MS system for the

length of a typical metabolomic sequence and the integrity of the samples in the autosampler during analysis.

3.2 Wine storage experiment

The method optimization described above was applied to an experimental design concerning wine storage. The aim of this project was to study how the metabolome of wine changes in two different conservation conditions over a period of 24 months: a) optimum storage conditions in a wine cellar, as compared to b) typical domestic storage. For the optimum storage conditions the temperature was kept stable at around ~16 ^{o}C during all seasons, and humidity was around 70%. In both cases the wines were kept in clean places, away from smells, light or heating systems and the bottles were in a horizontal position inside typical cardboard wine boxes. The latter parameters would seem appropriate for 'good' domestic storage, and are often also used in restaurants, wine stores, supermarkets and wine distributors, and are certainly not extreme. The only difference for domestic storage was that neither humidity nor temperature were controlled: temperature was around 20 ^{o}C during the winter and between 20 and 27 ^{o}C in the summer period. To increase the biological variability of the sample set, 5 different red wines were used and a different bottle was sampled for each time point/storage (reference or time zero, 6, 12, 18 and 24 months). Figure 3 shows a PCA score plot of the HILIC-MS results, based on 6311 features recognised using XCMS. The QCs cluster in the middle of the plot, except for the first 4 QC injections required for column equilibration. This tight QC cloud is proof of good system stability over the whole length of analysis. QC injection distribution/variability is the most popular and reliable method for controlling the quality of the untargeted metabolomics dataset (Arapitsas et al., 2014; Franceschi et al., 2014; Gika, Theodoridis, et al., 2014; Godzien, Alonso-Herranz, Barbas, & Armitage, 2014; Sangster, Major, Plumb, Wilson, & Wilson, 2006), and it has

recently also been introduced to targeted analysis (Ehrhardt, Arapitsas, Stefanini, Flick, & Mattivi, 2014). In addition, the manual integration of 50 selected peaks (including all markers in Table 1) with TargetLynx showed excellent stability in terms of retention time (less than 1 %CV) and peak area (less than 20 %CV). Secondly, there was a clear time trend for wine stored with home/domestic conservation in PC4 (horizontally). There was considerable variability for the five different wines and this was largely seen in PC1. As regards the effect of storage conditions, domestic conservation caused larger overall changes. The wines stored in optimum conditions remained relatively close to the time zero samples, while the domestically stored wines were widely distributed and the 18 and 24 months time points in particular were clearly further apart from the previous time points (12 and 6 months). This was expected on the basis of our previous study of the same wines with reverse phase LC-MS analysis (Arapitsas et al., 2014), also providing proof of the validity and utility of the method.

To identify features differentiating the two storage conditions, the OPLS-DA discrimination analysis tool of SIMCA P (Umetrics, Umea, Sweden) software was applied to the peak table from XCMS analysis. OPLS-DA analysis highlighted 81 out of a total 6311 features as tentative markers for the two conditions (with Variable Importance in the Projection or VIP >2). Manual integration of these 81 features from the raw file data using TargetLynx allowed to remove redundancy, decreasing the number of tentative markers to 34. This step helped to detect false positives by applying independent evaluation of the markers selected through XCMS and OPLS-DA, since it was applied to raw data. The manually integrated areas made it possible to confirm or reject the statistical significance of each marker in distinguishing the two storage conditions; and to evaluate the instrumental variability of each specific marker during the analysis by using the QCs injections. Another way of eliminating false positives is visual inspection of the markers' MS spectrum and LC chromatogram. The confirmed 34

421 feature-markers correspond to 29 metabolites, since in this list one compound had four
422 features and a second compound two features (Table 1). Of these tentative markers,
423 phenolic compounds known (Arapitsas et al., 2014; Kallithraka, Salacha, & Tzourou,
424 2009; Monagas et al., 2005; Wirth et al., 2010) to be influenced by storage length and
425 conditions were identified; these included quercetin, catechin, malvidin 3-glucoside and
426 pyranomalvidin 3-glucoside. Quercetin was higher for domestic storage as the product of
427 the quercetin 3-glucoside hydrolysis in these conditions, while the other three were
428 higher in the case of optimum cellar storage, in agreement with previous work by our
429 group, where they were found to be more stable at lower temperatures (Arapitsas et al.,
430 2014). In our RPLC-MS study, carried out with the same sample set, these four
431 metabolites together with pantothenic acid (Figure 4) were found to be influenced by the
432 storage conditions of the specific sample set. Moreover, the datasets obtained by the
433 two platforms, RPLC-MS and HILIC-MS, shared 12 common features with significant
434 differences for the two storage conditions (Table 1 and Arapitsas et al., 2014). Markers
435 eluting in the solvent front in the RPLC study were retained by the HILIC column and
436 vice versa. This confirmed the robustness of the instrumental set-up and data analysis
437 parameters used in both studies, but also the complementarity of the two platforms.
438 Using HILIC-MS mode a degradation product of malvidin 3-glucoside was also
439 shown to be higher for domestic storage (Table 1). Based on mass accuracy, isotopic
440 distribution and other m/z ions of the same peak (see Table 1), we tried to provide the
441 most probable molecular formula for all the ‘unknown’ metabolite markers. Although
442 almost 250 polar commercial standards were injected into the LC-MS system (Shahaf et
443 al., 2013) including the metabolites of the targeted metabolomics paper (Gika et al.,
444 2012), the number of unknown markers was 21 (from 29). Since the injected chemical
445 standard – reference material - list includes almost all commercial available polar
446 primary metabolites (carbohydrates, amino acids, organic acids, etc.) and many polar

447 secondary metabolites known to be present in wine, it could be concluded that the
448 metabolic space of wine is much bigger than is currently known and deserve further
449 investigation. This is not surprising if we consider that wine is one of the most complex
450 foods as far as the metabolomic profile is concerned, since grapes, yeasts, bacteria,
451 fungi, exogenous antioxidants, fining agents and other oenological materials and ageing
452 are involved in its preparation.

453 Different primary/secondary polar metabolites, including glucose, proline,
454 hypoxanthine, xanthine, xanthosine, salicylic acid, hexoses, inositols, phenylalanine,
455 betaine, citruline, uracil, choline, GABA, etc, did not show any statistically significant
456 variation (data not shown). From the molecular formulas (Table 1) it becomes clear that
457 most of the differentiating metabolites were nitrogen-contained (e.g. amines). The most
458 promising marker was the peak with a retention time of 18.37 min, since it has one of the
459 highest VIP values and 4 features in Table 1. This metabolite, identified (1^{st} level
460 identification) as 4-amino-heptanedioic acid (Figure 4), should not be confused with its
461 isomer 2-aminopimelic acid, which eluted 0.5 min later in our chromatographic system.
462 The ethyl ester of 4-amino-heptanedioic acid was another metabolite marker, higher in
463 cellar conditions. There were two peaks with the same *m/z* and isotopic distribution
464 between the markers (unknowns 12 and 16), which could correspond to the ethyl ester
465 of 4-amino-heptanedioic acid, but only one had 3 ions similar to the acid. The correlation
466 between 4-amino-heptanedioic acid and unknown 12 (tentatively identified as an ethyl
467 ester of 4-amino-heptanedioic acid) across all samples was 0.76, but only 0.22 with
468 unknown 16. On the other hand, unknown 16 had a correlation of 0.81 with pantothenic
469 acid, but is probably just a fragment of the main ion, since the loss of one oxygen should
470 not give a more polar metabolite. Table 1 reports the *m/z* and retention times of all
471 markers and some extra information (fragments, coelution ions, molecular formulas, etc)
472 which could be interesting for future work in the wine metabolomic field. Although some

additional hypotheses about the structure could be made, in the light of the high possibility of false positive error when the retention time is unknown (Arapitsas, Langridge, Mattivi, & Astarita, 2014), it was preferred not to speculate further about the possible molecular form of the unknown markers. In Figure 4 the kinetic graphs of pantothenic acid and 4-amino-heptanedioic acid and their chemical structure are shown. The concentration (expressed in area) of both markers decreased much faster in domestic conditions. The vitamin pantothenic acid concentration in foodstuff is known to be influenced by storage conditions and especially by temperature. On the other hand, the literature lacks information about 4-amino-heptanedioic acid, not only as regards its behaviour in food in various storage conditions, but in general in relation to its importance, while this is the first time 4-amino-heptanedioic acid has been found in wine, to our knowledge.

4. Conclusions

In conclusion, it was shown that a good starting pointing for developing a good and robust untargeted HILIC LC-MS method was a similar targeted method already validated for its functionality. By applying this idea, a HILIC-MS metabolomics method for wine polar metabolites was developed. The majority of the chromatographic parameters were unchanged and for the adaptation only eluent preparation was optimized in order to have constant mobile phase ionic strength. The other key point optimized was sample preparation, with the intention of ensuring instrumental stability for long batches in terms of chromatographic parameters (peak resolution and retention time) and MS accuracy and sensitivity. Although HILIC is considered less robust as compared to RPLC, here it was possible to have comparable results. Finally the influence of storage conditions on wine metabolic space was also confirmed for polar

499 metabolites and especially nitrogen organic compounds. 4-amino-heptanedioic acid and
500 its ethyl ester were detected for the first time in wine and were strong markers for
501 distinguishing wine stored in optimum conditions.
502
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508
509
510 **Literature**

511 Arapitsas, P., James, L., Fulvio, M., & Giuseppe, A. (n.d.). A Facile Database Search
512 Engine for Metabolite Identification and Biomarker Discovery in Metabolomics : :
513 Waters. Retrieved July 31, 2014, from
514 http://www.waters.com/waters/library.htm?locale=en_US&lid=134796033&cid=5114
515 36

516 Arapitsas, P., Scholz, M., Vrhovsek, U., Di Blasi, S., Biondi Bartolini, A., Masuero, D., …
517 Mattivi, F. (2012). A Metabolomic Approach to the Study of Wine Micro-
518 Oxygenation. (D. J. Kliebenstein, Ed.)*PLoS ONE*, *7*(5), e37783.
519 doi:10.1371/journal.pone.0037783

520 Arapitsas, P., Speri, G., Angeli, A., Perenzoni, D., & Mattivi, F. (2014). The influence of
521 storage on the “chemical age” of red wines. *Metabolomics*. doi:10.1007/s11306-
522 014-0638-x

523 Baker, M. (2011). Metabolomics: from small molecules to big ideas. *Nature Methods*,
524 *8*(2), 117–121. doi:10.1038/nmeth0211-117

525 Castro, C. C., Martins, R. C., Teixeira, J. A., & Silva Ferreira, A. C. (2014). Application of
526 a high-throughput process analytical technology metabolomics pipeline to Port wine
527 forced ageing process. *Food chemistry*, *143*, 384–91.
528 doi:10.1016/j.foodchem.2013.07.138

529 Cubero-Leon, E., Peñalver, R., & Maquet, A. (2014). Review on metabolomics for food
530 authentication. *Food Research International*, *60*, 95–107.
531 doi:10.1016/j.foodres.2013.11.041

532 Dalgliesh, C. E., Horning, E. C., Horning, M. G., Knox, K. L., & Yarger, K. (1966). A gas-
533 liquid-chromatographic procedure for separating a wide range of metabolites
534 occuring in urine or tissue extracts. Retrieved from
535 http://www.biochemj.org/bj/101/0792/bj1010792_browse.htm

536 Dunn, W. B., Erban, A., Weber, R. J. M., Creek, D. J., Brown, M., Breitling, R., … Viant,
537 M. R. (2012). Mass appeal: metabolite identification in mass spectrometry-focused
538 untargeted metabolomics. *Metabolomics*, *9*(S1), 44–66. doi:10.1007/s11306-012-
539 0434-4

540 Dunn, W. B., & Hankemeier, T. (2013). Mass spectrometry and metabolomics: past,
541 present and future. *Metabolomics*, *9*(S1), 1–3. doi:10.1007/s11306-013-0507-z

542 Ehrhardt, C., Arapitsas, P., Stefanini, M., Flick, G., & Mattivi, F. (2014). Analysis of the
543 phenolic composition of fungus-resistant grape varieties cultivated in Italy and
544 Germany using UHPLC-MS/MS. *Journal of mass spectrometry : JMS*, *49*(9), 860–9.
545 doi:10.1002/jms.3440

546 Franceschi, P., Mylonas, R., Shahaf, N., Scholz, M., Arapitsas, P., Masuero, D., …
547 Wehrens, R. (2014). MetaDB a Data Processing Workflow in Untargeted MS-Based
548 Metabolomics Experiments. *Frontiers in bioengineering and biotechnology*, *2*, 72.
549 doi:10.3389/fbioe.2014.00072

550 Ghidossi, R., Poupot, C., Thibon, C., Pons, A., Darriet, P., Riquier, L., … Mietton
551 Peuchot, M. (2012). The influence of packaging on wine conservation. *Food*
552 *Control*, *23*(2), 302–311. doi:10.1016/j.foodcont.2011.06.003

553 Gika, H. G., Theodoridis, G. A., Plumb, R. S., & Wilson, I. D. (2014). Current practice of
554 liquid chromatography-mass spectrometry in metabolomics and metabonomics.
555 *Journal of pharmaceutical and biomedical analysis*, *87*, 12–25.
556 doi:10.1016/j.jpba.2013.06.032

557 Gika, H. G., Theodoridis, G. A., Vrhovsek, U., & Mattivi, F. (2012). Quantitative profiling
558 of polar primary metabolites using hydrophilic interaction ultrahigh performance
559 liquid chromatography-tandem mass spectrometry. *Journal of chromatography. A*,
560 *1259*(null), 121–7. doi:10.1016/j.chroma.2012.02.010

561 Gika, H. G., Wilson, I. D., & Theodoridis, G. A. (2014). LC-MS-based holistic metabolic
562 profiling. Problems, limitations, advantages, and future perspectives. *Journal of*
563 *chromatography. B, Analytical technologies in the biomedical and life sciences*,
564 *966*, 1–6. doi:10.1016/j.jchromb.2014.01.054

565 Godzien, J., Alonso-Herranz, V., Barbas, C., & Armitage, E. G. (2014). Controlling the
566 quality of metabolomics data: new strategies to get the best out of the QC sample.
567 *Metabolomics*. doi:10.1007/s11306-014-0712-4

González Marco, A., & Ancín Azpilicueta, C. (2006). Amine concentrations in wine stored in bottles at different temperatures. *Food Chemistry*, *99*(4), 680–685. doi:10.1016/j.foodchem.2005.08.043

Gougeon, R. D., Lucio, M., Frommberger, M., Peyron, D., Chassagne, D., Alexandre, H., … Schmitt-Kopplin, P. (2009). The chemodiversity of wines can reveal a metabologeography expression of cooperage oak wood. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(23), 9174–9. doi:10.1073/pnas.0901100106

Hernanz, D., Gallo, V., Recamales, Á. F., Meléndez-Martínez, A. J., González-Miret, M. L., & Heredia, F. J. (2009). Effect of storage on the phenolic content, volatile composition and colour of white wines from the varieties Zalema and Colombard. *Food Chemistry*, *113*(2), 530–537. doi:10.1016/j.foodchem.2008.07.096

Holčapek, M., Jirásko, R., & Lísa, M. (2012). Recent developments in liquid chromatography-mass spectrometry and related techniques. *Journal of chromatography. A*, *1259*, 3–15. doi:10.1016/j.chroma.2012.08.072

Hopfer, H., Ebeler, S. E., & Heymann, H. (2012). The Combined Effects of Storage Temperature and Packaging Type on the Sensory and Chemical Properties of Chardonnay. *Journal of Agricultural and Food Chemistry*, *60*(43), 10743–10754. doi:10.1021/jf302910f

Kallithraka, S., Salacha, M. I., & Tzourou, I. (2009). Changes in phenolic composition and antioxidant activity of white wine during bottle storage: Accelerated browning test versus bottle storage. *Food Chemistry*, *113*(2), 500–505. doi:10.1016/j.foodchem.2008.07.083

Loscos, N., Hernández-Orte, P., Cacho, J., & Ferreira, V. (2010). Evolution of the aroma composition of wines supplemented with grape flavour precursors from different varietals during accelerated wine ageing. *Food Chemistry*, *120*(1), 205–216. doi:10.1016/j.foodchem.2009.10.008

Mattivi, F., Arapitsas, P., Biondi Bartolini, A., Di Blasi, S., Perenzoni, D., Rigo, A., & Vrhovsek, U. (2012). *“Il primo approccio metabolomico per lo studio della micro-ossigenazione” in La ricerca applicata ai vini di qualità.* (p. 216). Firenze University Press. Retrieved from http://books.google.com/books?id=myHDc51q5mQC&pgis=1

Monagas, M., Bartolomð, B., & Gðmez-Cordovðs, C. (2005). Evolution of polyphenols in red wines from Vitis vinifera L. during aging in the bottle. *European Food Research and Technology*, *220*(3-4), 331–340. doi:10.1007/s00217-004-1109-9

Nicholson, J. K., & Lindon, J. C. (2008). Systems biology: Metabonomics. *Nature*, *455*(7216), 1054–6. doi:10.1038/4551054a

Noack, S., & Wiechert, W. (2014). Quantitative metabolomics: a phantom? *Trends in biotechnology*, *32*(5), 238–44. doi:10.1016/j.tibtech.2014.03.006

606 Oms-Oliu, G., Odriozola-Serrano, I., & Martín-Belloso, O. (2013). Metabolomics for
607 assessing safety and quality of plant-derived food. *Food Research International*,
608 *54*(1), 1172–1183. doi:10.1016/j.foodres.2013.04.005

609 Sangster, T., Major, H., Plumb, R., Wilson, A. J., & Wilson, I. D. (2006). A pragmatic and
610 readily implemented quality control strategy for HPLC-MS and GC-MS-based
611 metabonomic analysis. *The Analyst*, *131*(10), 1075–8. doi:10.1039/b604498k

612 Shahaf, N., Franceschi, P., Arapitsas, P., Rogachev, I., Vrhovsek, U., & Wehrens, R.
613 (2013). Constructing a mass measurement error surface to improve automatic
614 annotations in liquid chromatography/mass spectrometry based metabolomics.
615 *Rapid Communications in Mass Spectrometry*, *27*(21), 2425–2431.
616 doi:10.1002/rcm.6705

617 Spagou, K., Wilson, I. D., Masson, P., Theodoridis, G., Raikos, N., Coen, M., … Want,
618 E. J. (2011). HILIC-UPLC-MS for exploratory urinary metabolic profiling in
619 toxicological studies. *Analytical chemistry*, *83*(1), 382–90. doi:10.1021/ac102523q

620 T'kindt, R., Storme, M., Deforce, D., & Van Bocxlaer, J. (2008). Evaluation of hydrophilic
621 interaction chromatography versus reversed-phase chromatography in a plant
622 metabolomics perspective. *Journal of separation science*, *31*(9), 1609–14.
623 doi:10.1002/jssc.200700539

624 Theodoridis, G., Gika, H., Franceschi, P., Caputi, L., Arapitsas, P., Scholz, M., … Mattivi,
625 F. (2011). LC-MS based global metabolite profiling of grapes: solvent extraction
626 protocol optimisation. *Metabolomics*, *8*(2), 175–185. doi:10.1007/s11306-011-0298-
627 z

628 Valls, J., Millán, S., Martí, M. P., Borràs, E., & Arola, L. (2009). Advanced separation
629 methods of food anthocyanins, isoflavones and flavanols. *Journal of*
630 *chromatography. A*, *1216*(43), 7143–72. doi:10.1016/j.chroma.2009.07.030

631 Vilhena, R. de O., Pontes, F. L. D., Marson, B. M., Ribeiro, R. P., Carvalho, K. A. T. de,
632 Cardoso, M. A., & Pontarolo, R. (2014). A new HILIC-MS/MS method for the
633 simultaneous analysis of carbidopa, levodopa, and its metabolites in human
634 plasma. *Journal of chromatography. B, Analytical technologies in the biomedical*
635 *and life sciences*, *967C*, 41–49. doi:10.1016/j.jchromb.2014.06.030

636 Willemse, C. M., Stander, M. A., & De Villiers, A. (2013). Hydrophilic interaction
637 chromatographic analysis of anthocyanins. *Journal of chromatography. A*, *1319*,
638 127–40. doi:10.1016/j.chroma.2013.10.045

639 Wirth, J., Morel-Salmi, C., Souquet, J. M., Dieval, J. B., Aagaard, O., Vidal, S., …
640 Cheynier, V. (2010). The impact of oxygen exposure before and after bottling on the
641 polyphenolic composition of red wines. *Food Chemistry*, *123*(1), 107–116.
642 doi:10.1016/j.foodchem.2010.04.008

643 Wu, H., Southam, A. D., Hines, A., & Viant, M. R. (2008). High-throughput tissue
644 extraction protocol for NMR- and MS-based metabolomics. *Analytical biochemistry*,
645 *372*(2), 204–12. doi:10.1016/j.ab.2007.10.002

Zhu, R.-H., Li, H.-D., Cai, H.-L., Jiang, Z.-P., Xu, P., Dai, L.-B., & Peng, W.-X. (2014). Validated HILIC-MS/MS assay for determination of vindesine in human plasma: Application to a population pharmacokinetic study. *Journal of pharmaceutical and biomedical analysis*, *96*, 31–6. doi:10.1016/j.jpba.2014.03.017

Figure Captions

Figure 1. A) The trend in the number of features for the pooled sample (QC) after 20 injections and in different dilutions with acetonitrile. B) LC-MS stability test of the optimised sample preparation for 55 consecutive injections.

Figure 2. Selected LC-MS chromatographic graphs for different compounds of the same sample (QC) in various dilutions with acetonitrile (not diluted wine, 1:1, 1:2, 1:4, 1:6 and 1:9 v:v wine:acetonitrile).

Figure 3. PCA plot of kinetic experiment analysis in the two storage conditions tested. The wines stored in optimum cellar conditions are shown with black dots, wines stored in typical domestic conditions in red, the reference wines in blue, and the QC samples in green. The numbers indicate the length of storage in months (0, 6, 12, 18 and 24 months).

Figure 4. Average change, based in 5 different red wines, of 4-amino-heptanedioic (a) and acid pantothenic acid (b) during storage in the two conditions (cellar **----** vs. domestic **- - -**). Areas correspond to integrated peaks calculated using TargetLynx.

Table(s)

Table 1. Features markers for wine storage experiment (cellar vs domestic) together with their annotation, LC-MS and statistical information.

m/z	rt (min)	Higher in	VIP[+]	Annotation	Formula	Theoretical m/z	error m/z (ppm)	Comments
186.1131	1.02	domestic	3.08	Unknown 1	C9H15NO3			MS/MS: 170.04
216.1073[#]	1.31	cellar	3.76	Unknown 2				together with m/z 160.1306; MS/MS: 143.04 and 115.02
303.0500	1.33	domestic	3.62	quercetin*	C15H10O7	303.0499	-0.25	[M+H]+
174.1494	2.27	domestic	3.01	Unknown 2	C9H19NO2	174.1494	0.00	together with m/z 87.0454
234.0803	2.32	cellar	2.77	Unknown 3				
111.0558	2.42	domestic	2.92	Unknown 4	C5H6N2O	111.0558	0.00	MS/MS: 82.0526
166.0509	3.14	domestic	6.43	Unknown 5	C8H7NO3	166.0504	-3.01	
291.0877[#]	3.22	cellar	3.07	catechin*	C15H14O6	291.0863	-4.78	[M+H]+
347.0771[#]	3.58	domestic	2.83	syringetin*	C17H14O8	347.0761	-2.88	together with 509.1231; [M-glucose+H]+
317.0797	3.8	domestic	2.21	Unknown 7	C18H10N3O3	317.0800	0.95	together with m/z 214.0173
97.0289	4.11	domestic	2.98	Unknown 8	C5H4O2	97.0290	1.03	
136.0620	4.26	cellar	8.33	Unknown 9	C5H3N4+NH3	136.0623	2.20	together with m/z 119.036 and 94.0404; MS/MS: 119.04 and 94.04
169.0511	4.39	domestic	6.75	Unknown 10	C9H4N4	169.0514	1.77	MS/MS: 150.05
184.0640	4.51	cellar	4.83	Unknown 11	C5H13NO4S	184.0643	1.63	together with m/z 104.1073; Sulfonated C5H13NO; MS/MS: 104.10 and 60.05
333.0975[#]	6.67	domestic	3.24	malvidin derivative[‡]	C17H16O7	333.0969	-1.86	together with m/z 495.1483 and 315.0884
204.1241[#]	9.21	cellar	5.39	Unknown 12	C9H17NO4	204.1236	-2.45	MS/MS: 158.09, 112.07; 4-aminoheptanedioic acid ethyl ester
349.0925	9.44	cellar	4.34	Unknown 13	C11H20N6OS3	349.0939	4.01	MS/MS: 317.18, 181.04, 139.03, 120.06
220.119[#]	9.61	cellar	2.93	pantothenic acid*	C9H17NO5	220.1179	-4.80	[M+H]+
232.1188	10.55	domestic	4.60	Unknown 14				MS/MS: 72.02, 85.01, 103.02
493.134[#]	10.72	cellar	5.80	malvidin 3-glucoside*	C23H25O12	493.1341	0.11	[M+]
363.0685	12.86	cellar	2.72	Unknown 15				MS/MS: 136.03
347.0949	12.87	cellar	3.17	Unknown 15				
138.0549	13.11	cellar	8.95	amino-Benzoic acid	C7H7NO2	138.0555	4.35	[M+H]+
204.1236[#]	13.91	cellar	5.40	Unknown 16	C9H17NO4	204.1236	0.00	MS/MS: 165.09, 145.05, 85.00
347.0954	14.05	domestic	4.63	Unknown 17				
561.1239[#]	18.15	cellar	2.24	vitisin A*	C26H25014	561.1239	-0.03	[M]+
176.0923[#]	18.37	cellar	6.98	4-aminoheptanedioic acid*	C7H13NO4	176.0923	0.00	[M+H]+
158.0817[#]	18.37	cellar	4.12	4-aminoheptanedioic acid*	C7H11NO3	158.0817	0.00	[M-H2O+H]+
130.0862	18.37	cellar	4.09	4-aminoheptanedioic acid*	C6H11NO2	130.0868	4.61	[M-H2O-CO+H]+
112.0757[#]	18.37	cellar	3.94	4-aminoheptanedioic acid*	C6H9NO	112.0762	4.46	[M-H2O-CO-H2O+H]+
344.0912[#]	20.49	cellar	7.78	Unknown 18	C11H20KN4O4S	344.0921	2.62	305.1739 and 163.0404; MS/MS: 241.01, 197.01 and 153.01
212.0564	21.78	domestic	3.00	Unknown 19				
282.1013	22.22	cellar	7.09	Unknown 20				MS/MS: 156.05 and 117.02
286.1403	24.51	domestic	5.09	Unknown 21	C12H19N3O5	286.1403	0.00	together with 185.0928; MS/MS: 185.09 and 100.02

[‡]malvidin 3-glucoside degration product after hydrogenation; rt: retention time: [+]VIP: Variable Influence on Projection (cellar versus domestic storage). [#] features also markers in the RP LC-MS experiment (Arapitsas et al. 2014). Unknowns with the same number are features which belong to the same peaks. *1[st] level annotation (confirmed by authentic standard).

Figure(s)

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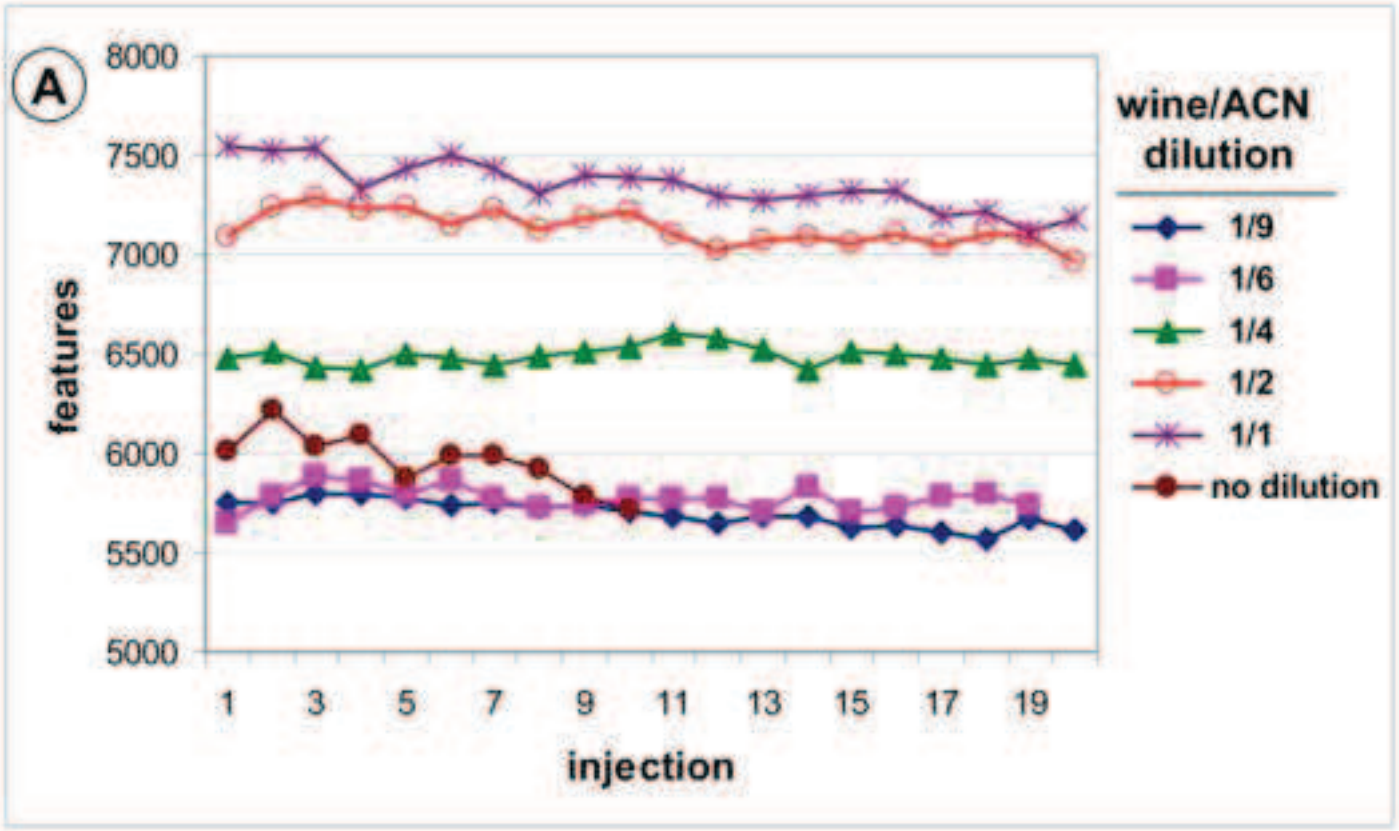

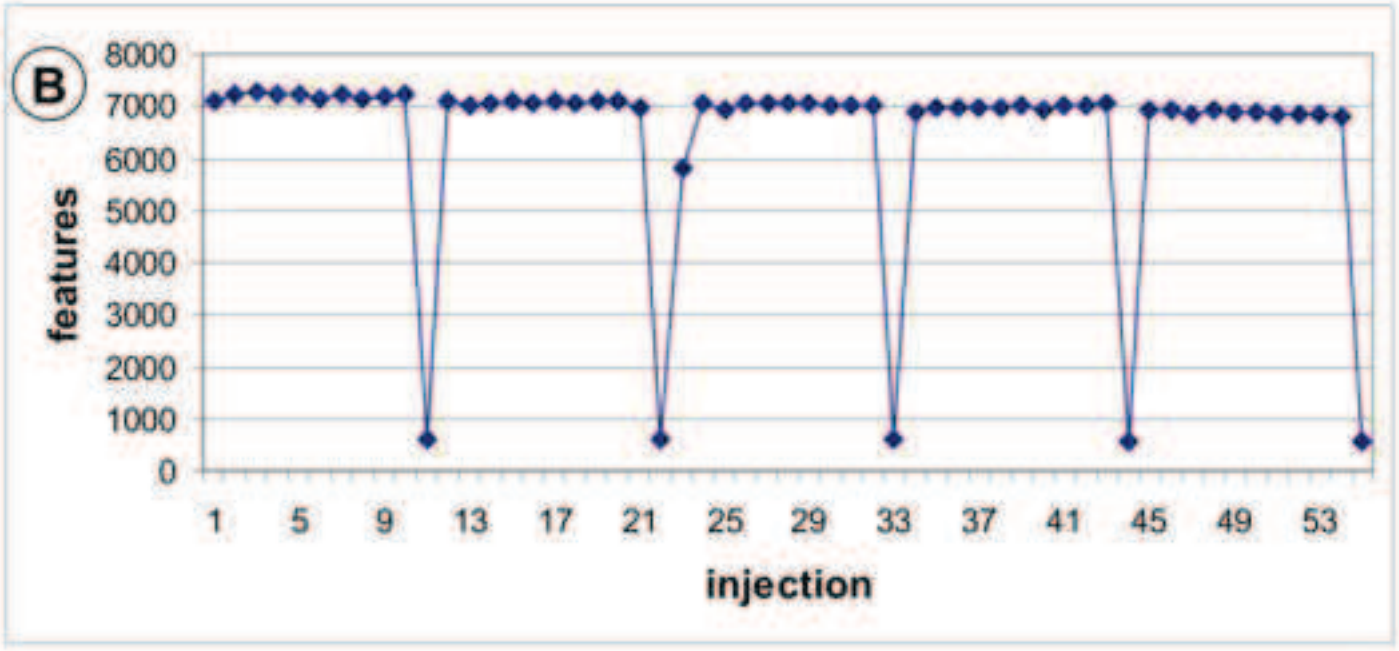

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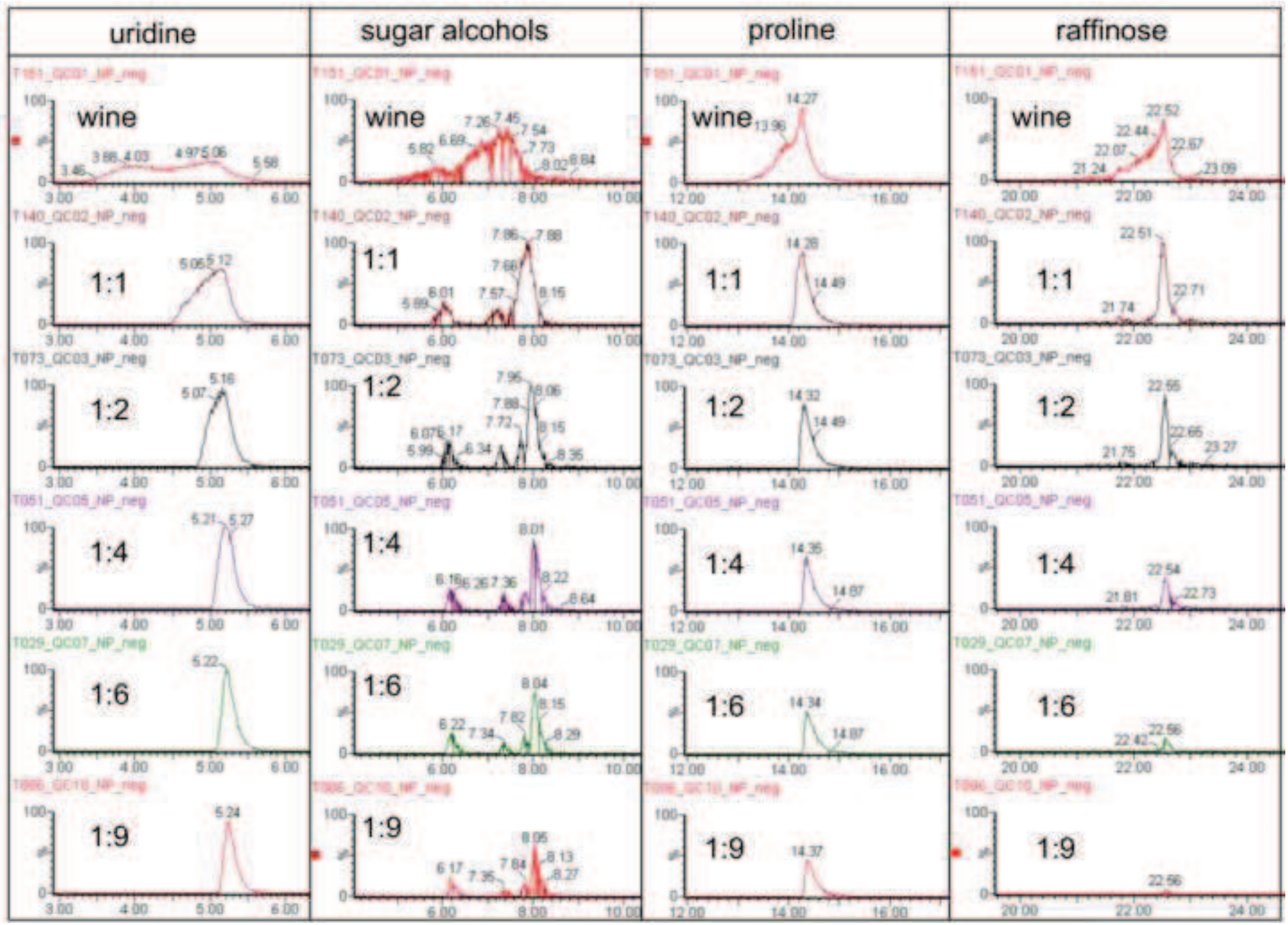

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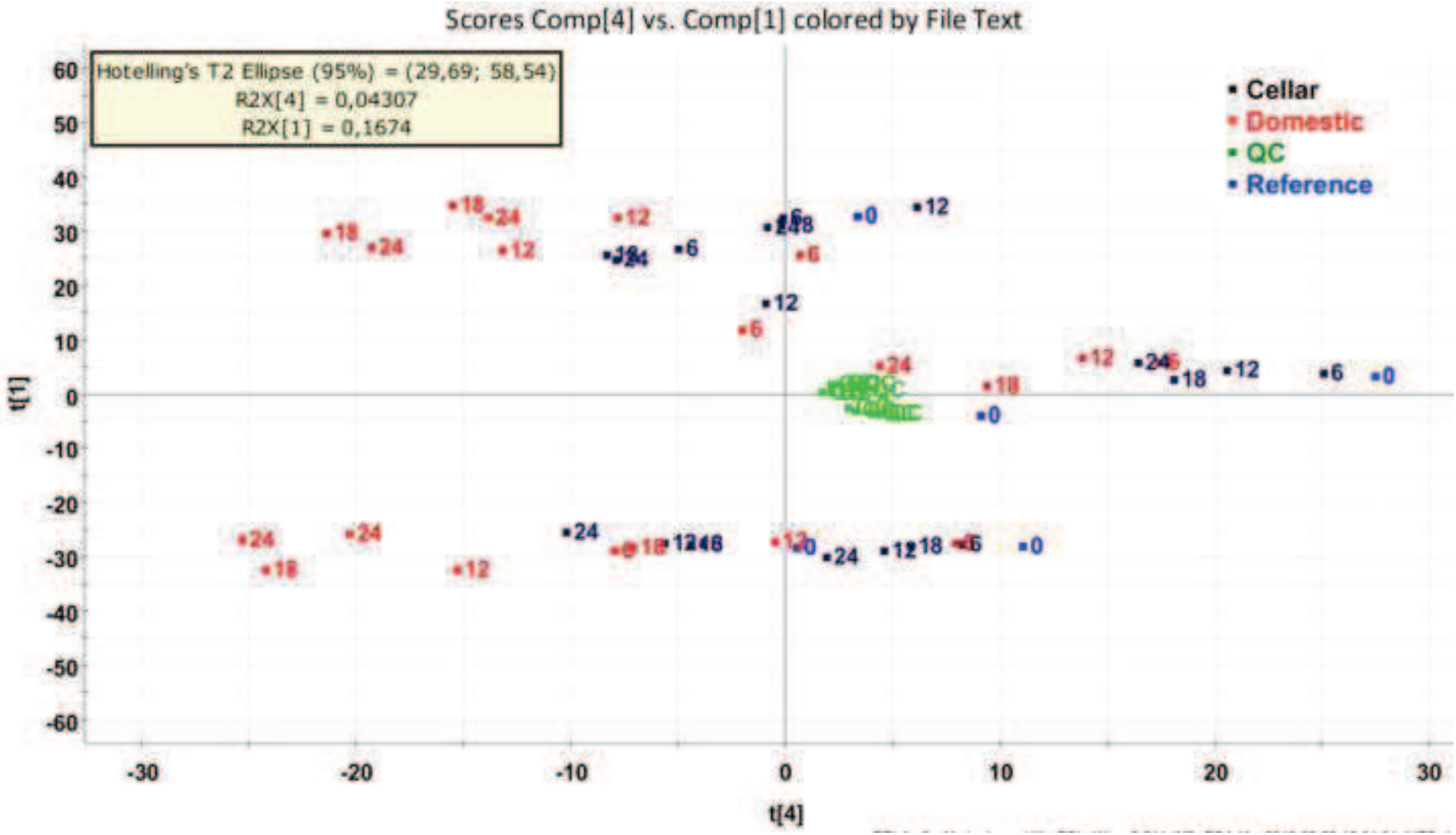

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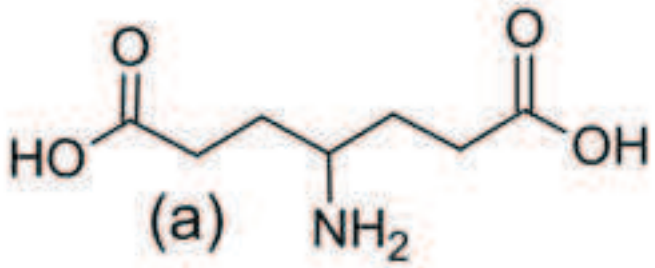

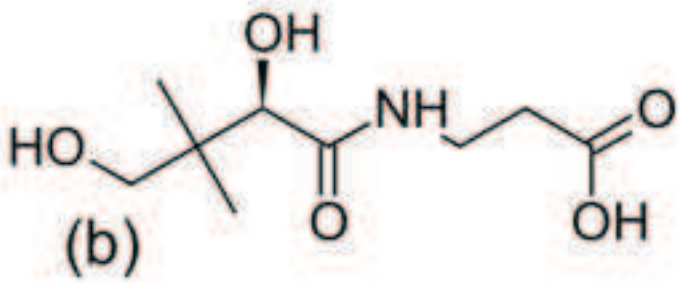

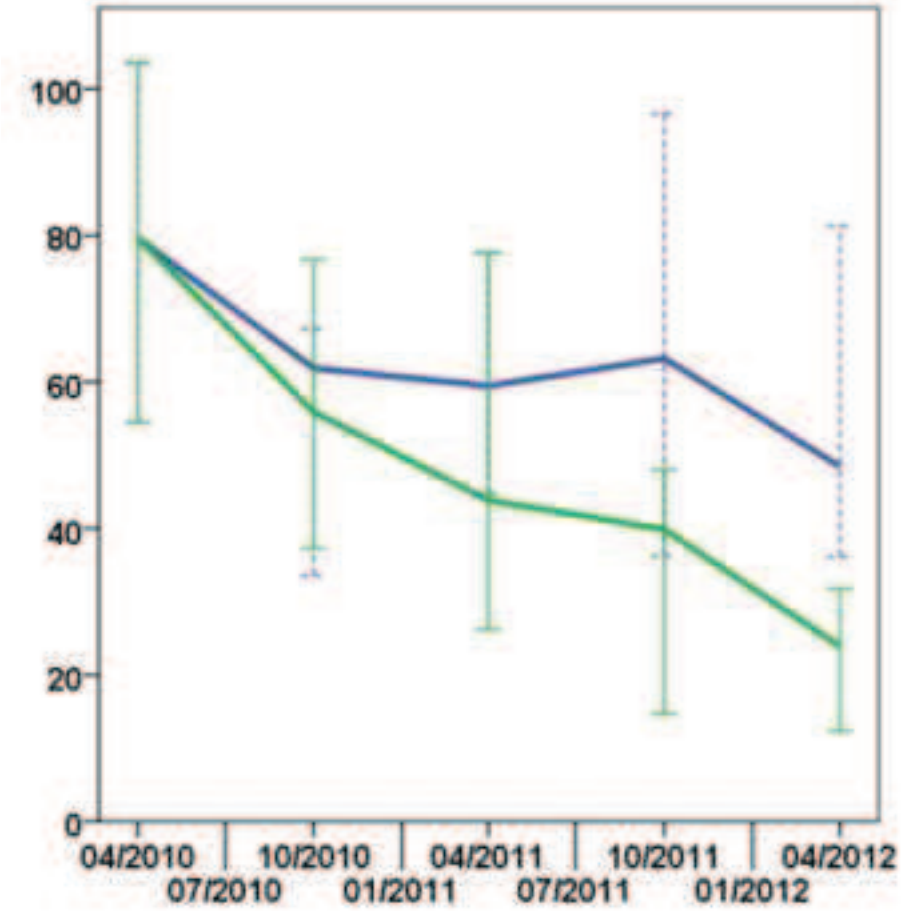

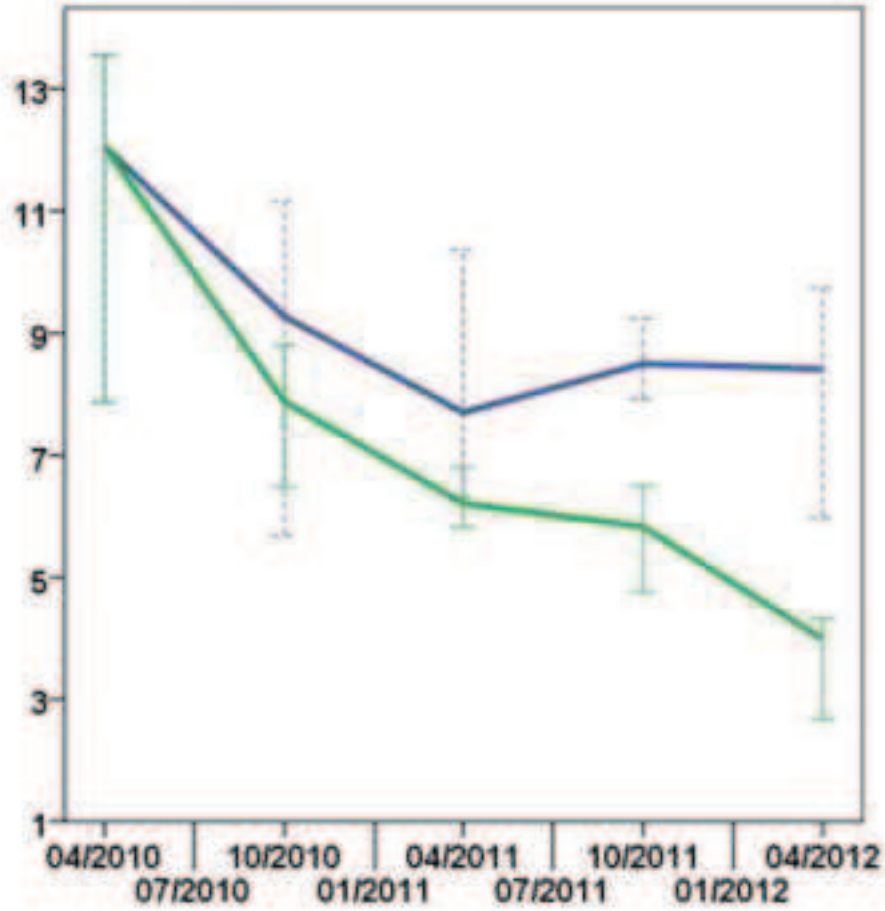

Studying the effect of storage conditions on the metabolite content of red wine using HILIC LC-MS based metabolomics

Highlights

- Adaptation of a targeted HILIC-MS method to untargeted metabolomics
- HILIC-MS untargeted method validation for wine metabolomics
- Slightly sub-optimum storage conditions had a major impact in wine metabolome
- 4-amino-heptanedioic acid was annotated for the first time in wine
- 4-amino-heptanedioic acid was a marker for wine sub-optimum storage conditions