Tandem HILIC-RP Liquid Chromatography for Increased Polarity Coverage in Food Analysis

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List of abbreviations

ANP	Aqueous normal phase
AS	Autosampler
P1	Pump 1
P2	Pump 2
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet detector

Keywords

Foodomics | LC-MS | Non-target analysis | Tandem liquid chromatography | Wine

Abstract

Comprehensive non-targeted analysis of food products normally requires two complementary chromatographic runs to achieve maximum compound coverage. In this study, we present a sensitive tandem-LC method, which combines RP and HILIC separation in a single run. The setup consists of a C18 trap column and two subsequently coupled analytical columns (HILIC and C18) which are operated in parallel. First, hydrophobic compounds are retained on the RP trap column while rather hydrophilic compounds are directly transferred onto a HILIC phase. Next, the pre-fractionated sample composition is analyzed by HILIC or RP chromatography, respectively. The presented setup allows individual and independent gradient elution as well as interfacing with mass spectrometry. The performance of the method has been proven by means of food relevant standards and analysis of complex food samples (*e.g.* red wine, meat extract). The simple and robust setup provides high flexibility in the selection of column combinations and does not require sophisticated instrumental setups or software. The method significantly increases the covered polarity range compared to classical one-dimensional chromatography. Our results indicate that tandem LC is a valuable and universal tool in the non-targeted screening of various types of complex food samples.

1 Introduction

The integration of biotic and abiotic processes makes foods very complex chemical systems. For example, the chemical complexity of wine combines biochemical impacts, such as plant, microorganism and secondary metabolisms, with environmental and viticultural impacts [1, 2]. Typical small molecular constituents of wine are primary metabolites (*e.g.* carbohydrates, organic acids, lipids, and amino acids) and phytochemicals (*e.g.* phenols, lignans, sterols, and alkaloids) [3, 4]. It is assumed that secondary metabolism leads to more than 200,000 natural products present in the plant kingdom [5]. Although application of metabolomics tools to food fingerprinting has rapidly emerged in recent years, only a small number of these compounds have been identified and listed in databases. Liquid chromatography coupled to high-resolution mass spectrometry (LC-MS) has prevailed as an excellent method in metabolomics [6, 7]. However, when aiming to identify as many compounds as possible, selection of an appropriate stationary phase is difficult because of a wide range of chemical and physical properties (*e.g.* polarity, pK_a) of the metabolites [6, 8]. Arubulu *et al.* have recently shown for red wine samples that a maximum metabolite coverage requires reversed-phase (RP) and hydrophilic liquid interaction chromatography (HILIC). While organic acids and carbohydrates were preferentially retained on the HILIC column, for most other compound classes no preferred separation phase could be found [3]. Two independent chromatographic runs, however, are time consuming and require larger sample amounts. Additionally, accurate alignment of unknown compounds that show retention on multiple stationary phases, such as RP and HILIC, is a challenging task.

To overcome these limitations, several setups have been developed which combine RP chromatography and HILIC in a single run. A first type of setup uses serial coupling of HILIC and RP columns [9–14]. Greco *et al.* showed an increased polarity range covered when a

zwitterionic HILIC column was serially coupled to a C18 RP column in the analysis of phenolic compounds [9]. However, serial coupling of columns does not allow independent gradient elution [15] and restricts the selection of columns due to an increase in back pressure. Good summaries of serial couplings have recently been published in comprehensive reviews by Haggarty and Burgess [16] and Alvarez-Segura *et al.* [17]. The second type of setup (tandem LC) uses first a trap column to divide the sample composition into hydrophilic and hydrophobic compounds. The pre-fractionated sample can then be analyzed on two different stationary phases (*e.g.* HILIC and RP). Due to the pre-fractionation step, analytes are separated either on the HILIC or on the RP column using independent gradient elution [15, 18]. Pyke *et al.* recently reported a tandem method, which combines aqueous normal phase (ANP) chromatography with RP separation for the analysis of urine metabolites [15]. They showed that the number of detected compounds using the tandem method is comparable to results when the same sample was analyzed in two independent chromatographic runs.

The aim of this study was to develop a HILIC-RP tandem coupling which allows analysis of highly polar to nonpolar food constituents in a single chromatographic run. Online pre-fractionation of the sample composition and subsequent analysis on two orthogonal stationary phases enhances the covered polarity range compared to traditional one-dimensional LC systems. Compared to serial couplings the here presented setup can be operated with individual gradients and bypasses limitations due to an increased column backpressure. By means of a second switching valve effluates of the two analytical columns enter the mass spectrometer individually, thus, avoiding possible loss in ionization efficiency.

2 Materials and Methods

2.1 Reagents

LC-MS grade methanol and acetonitrile (ACN) were purchased from Merck (Darmstadt, Germany). Formic acid (LC-MS grade) and ammonium formate (10 M stock solution) were obtained from Sigma-Aldrich (Steinheim, Germany). MilliQ-purified water (18.2 MΩ; Millipore, Germany) was used throughout the experiments.

2.2 Reference standards

Reference standards L-Alanine (99%), L-arginine (≥98%), L-asparagine monohydrate (>99%), L-aspartic acid (>98%), p-cresol (analytical standard), L-cysteine (>98%), D-(-)-fructose (>99%), fumaric acid (>99%), D-(+)-glucose (>99.5%), L-glutamic acid (>99%), L-glutamine (>99%), glycine (>99%), L-histidine (98%), L-isoleucine (99%), L-leucine (>99.5%), L-lysine (>98%), L-(-)-malic acid (>99.5%), L-methionine (>98%), myricetin (≥98%), L-phenylalanine (99%), L-proline (>99%), pyrocatechol (>99%), quercetin (≥95%), D-(-)-ribose (98%), L-serine (99%), succinic acid (≥99%), sucrose (>99.5%), syringic acid (analytical standard), L-threonine (>98%), D-(+)-trehalose dihydrate (>99%), L-tyrosine (>99%), and L-valine (>98%) were obtained from Sigma-Aldrich (Steinheim, Germany). Malvin chloride (Rotichrom® HPLC) was purchased from Roth (Karlsruhe, Germany) and malvidine chloride was purchased from Biomol (Hamburg, Germany). Individual stock solutions (1 mg·mL^{-1}) were prepared of each reference standard. Amino acid standards were dissolved in methanol/water (1:1) *v/v*, sugar and dicarboxylic acids were dissolved in water, and phenolic compounds were dissolved in methanol, respectively. Stock solutions were

diluted with water/ACN (98:2) *v/v* prior to injection. Concentration of the injected standards was 20 µg·ml^{-1} (amino acids: 10 µg·ml^{-1}), respectively.

2.3 Sample preparation

Red wine. A wine sample from the grape variety "Lemberger" with an alcohol content of 13.0% was diluted (1:5) *v/v* with water/ACN (98:2) *v/v* prior to injection.

Meat extract. 5 g minced beef were extracted in a blender with 30 ml methanol/water/hexane (1:1:1) *v/v/v* for 2.5 min. After centrifugation, 1 mL of the clear methanol/water-phase was vacuum dried and reconstituted in water/ACN (98:2) *v/v* before injection.

Brewed coffee (Italian espresso). 10 g freshly grinded coffee beans (100% Arabica beans) were brewed with 30 ml hot water. After centrifugation, the supernatant was diluted 1:10 with water/ACN (98:2) *v/v*.

Prior to each food sample, blank samples (water/ACN (98:2) *v/v)* were analyzed in order to subtract impurities and chemical noise from the sample results. Only features were reported which were exclusively found in food samples but not in the blank chromatograms.

2.4 Instrumental setup

Chromatographic analysis was carried out on a Thermo Scientific Dionex Ultimate 3000 system (Dreieich, Germany) equipped with two vacuum degassers, dual gradient pump (P1 and P2), a temperature controlled autosampler (AS), a thermostat controlled column oven containing two 10-port 2-position valves, and a variable wavelength detector (UV). The autosampler temperature was set to 5 °C. Column temperature was maintained at 40 °C. Connections were made using stainless-steel Viper capillaries (180 µm ID; Thermo Scientific,

Dreieich, Germany) with shortest possible lengths. A Kinetex C18 column (2.1 × 30 mm, 2.6 µm; Phenomenex, Aschaffenburg, Germany) was used for trapping hydrophobic analytes. For chromatographic separations, a ZIC-cHILIC column (2.1 × 100 mm, 3 µm, Merck, Darmstadt, Germany) and a Kinetex C18 column (2.1 × 100 mm, 2.6 µm, Phenomenex, Aschaffenburg, Germany) were used. Samples and blanks were injected in the LC flow stream via full-loop-injection (20 µl). The LC system was coupled to a Bruker maXis qTOF-MS equipped with an APOLLO II electrospray ion source (Bruker Daltonics, Bremen, Germany). Detection was run in both electrospray modes (ESI(-) and ESI(+)). Source settings in ESI(-) mode were: nebulizer pressure = 2 bar, dry gas flow = 10 $L \cdot min^{-1}$, dry gas temperature = 200 °C, capillary voltage = 4.0 kV, end plate offset = -500 V, mass range = 50 - 1500 *m/z*. Source settings in ESI(+) mode were: nebulizer pressure = 2 bar, dry gas flow = 10 $L \cdot min^{-1}$, dry gas temperature = 200 °C, capillary voltage = 4.5 kV, end plate offset = +500 V, mass range = 50 - 1500 *m/z*. The time-of-flight mass analyzer was calibrated by means of a commercial ESI Low Concentration Tune Mix (Agilent Technologies, Waldbronn, Germany). Additionally, prior to each chromatographic run, the same calibrants were injected for internal recalibration of each analyzed sample.

2.5 Chromatographic conditions

The RP mobile phase (P1) was a composition of 0.1% formic acid (solvent P1-A) and ACN + 0.1% formic acid (solvent P1-B). The HILIC mobile phase (P2) was a mixture of 5 mM ammonium formate/ACN (95:5) *v/v* (eluent P2-A) and ACN (eluent P2-B). Trapping phase (0-5 min) was run with isocratic conditions while RP and HILIC separations were run in gradient mode as shown in Table 1. Columns were re-equilibrated for 15 min after each chromatographic run to reach initial conditions.

2.6 Data processing

Raw data were post-processed with Genedata Expressionist for MS 11.0 (Genedata, Basel, Switzerland). After chromatogram smoothing and noise subtraction, mass spectra were internally calibrated. For internal calibration, the same Tune Mix as above was injected prior to each sample injection. Next, retention times were aligned to correct for shifts between chromatograms. Peak picking was done based on a curvature-based algorithm and heavy isotopes were identified and removed. The final data matrix consists of grouped chromatographic features (aligned *m/z*-values and retention times) and peak intensities. Original values, such as retention times and *m/z*-values were retained in the matrix and used for method validation purposes. All further data processing and statistics were done in Microsoft Excel 2016 and R Statistical Language (version 3.4.1) [19].

3 Results and Discussion

3.1 Principle setup

The here introduced tandem LC-system is build up of a short C18 RP-column (trap column) and two analytical separation columns (zwitterionic HILIC and C18 RP). HILIC phases are known to retain hydrophilic compounds (*e.g.* carbohydrates, amino acids) while RP columns have a complementary retention preference for nonpolar substances. In addition, Chalcraft *et al.* showed that the combination of HILIC and RP columns provides maximum orthogonality [11]. The system consists of three major steps: (i) loading/trapping phase (0-5 min), (ii) HILIC (5-25 min) and, (ii) RP separation (25–45 min). In a first step (0-5 min, Fig. 1A), the sample composition is passed through the trap column in isocratic mode. While hydrophobic analytes are retained on the C18 trap column more hydrophilic compounds are directly passed onto a

HILIC phase. A T-piece and addition of high organic mobile phase ensures the immediate binding of hydrophilic analytes on the HILIC column. The combined eluent composition during the trapping step at the HILIC column is 87.8% at a total flow rate of 400 µl·min^{-1}. A trapping time of five minutes (50 µl·min^{-1} flow rate) was found to be sufficient to remove most disturbing salts and other sample components, which cannot be separated neither by HILIC nor by RP. Two 10-port valves allow subsequent and independent chromatographic runs of the divided sample composition. First, compounds retained on the HILIC phase are separated running a linear gradient (5-25 min, Fig. 1B and Table 1). In this time, the composition of the pump 1 flow (passes trap column and RP separation column) is hold at initial conditions. We could not observe considerable band broadening when the flow of P1 was kept at initial conditions during HILIC separation. After the HILIC separation, analytes trapped on the C18 trap column are consecutively separated by a linear gradient (25–45 min, Fig. 1C and Table 1) by the two C18 RP columns. At the end of each run, the system is re-equilibrated to reach initial conditions. This type of instrumental setup allows two independent chromatographic runs, after dividing the analytes of a sample into a hydrophobic and hydrophilic part during the trapping phase, on two different analytical columns. By comparison, Pyke *et al.* used only one valve to switch between the three steps [15]. In their setup, effluates of the HILIC and RP column were continuously recombined by a T-piece before entering the detector. By using a second valve, it was possible to transfer the column effluates individually into the mass spectrometer. Hence, conditions in the ion source are the same as in single one-dimensional LC and loss in ionization efficiency can be avoided.

3.2 Analysis of food relevant reference standards

A selection of 34 food relevant reference standards was analyzed with the HILIC-RP tandem coupling method as described above (chromatographic conditions are given in Table 1). Standards included amino acids, carbohydrates, small dicarboxylic acids, and phenolic compounds covering a logP range from -3.5 to 2.1. Amino acids were injected with a concentration of 10 $\mu g \cdot ml^{-1}$. All other standards were analyzed with a concentration of 20 $\mu g \cdot ml^{-1}$, respectively. As shown in Fig. 2A rather hydrophilic compounds (amino acids, sugars, and dicarboxylates) were directly transferred onto the HILIC column during the loading phase and subsequently separated by HILIC. It is worth noting that succinate and ribose showed only weak retention on the HILIC phase and thus eluted already at the end of the trapping phase. By comparison, with except of pyrocatechol, all tested phenolic compounds were retained on the C18 trap column and consequently separated in the RP part. Although not all standards revealed baseline separated peaks, mass selective detection was able to resolve all tested standards with exception of isoleucine and leucine.

3.3 Online pre-fractionation of sample components (loading step)

The eluent composition during the trapping phase is crucial for the partitioning of sample analytes between the two analytical columns. The high chemical diversity [4, 20] and the simple and loss-free sample preparation (dilution) make wine an ideal food representative in the development and validation of non-targeted methods. Figure 2B shows chromatograms of a red wine sample analyzed by three different eluent compositions during the trapping phase (0–5 min). Increasing the organic content of the eluent of P1 increases also the organic content of the combined eluent composition at the HILIC phase, thus, enhances the retention of more hydrophilic compounds on the HILIC column. Increasing the organic content of P1, however,

counteracts with the capability of retaining hydrophobic compounds on the C18 trap column. Most equal distribution of analytes was achieved when the organic content was kept at a minimum (2.5% ACN, Fig. 2A). Increasing the ACN content of P1 reduced the number of analytes retained on the trap column dramatically. The number of features (S/N > 3) detected in ESI(-) after RP separation is halved when the ACN content of P1 is increased from 2.5% to 10%. Here, a feature is an analytical signal characterized by a unique retention time and ion mass [6, 21]. When using a P1-ACN content of 2.5% 463 ± 6 features could be detected after RP separation compared to 230 ± 2 at P1=10% ACN. Although the total number of detected features was very similar (1180 at P1=2.5% ACN, 1131 at P1=10% ACN) an uneven distribution of the analytes increases the number of co-eluting compounds, thus, complicating subsequent detection, *e.g.* by an increased chance of ion suppression in mass spectrometry. Moreover, when using a low ACN concentration during the trapping period, the number of weakly resolved signals in the first five minutes can be minimized. In this region of the chromatogram highest effects of ion suppression must be expected [11]. Signals eluting during the first five minutes result from compounds that are not or only weakly retained on the C18 trap column and the HILIC column. A P1-ACN content of 25% was found to be already high enough to minimize wine analytes retained on the trap column to less than 10% (37 ± 5 detected features) compared to P1=2.5% ACN (463 ± 6).

3.4 Non-targeted analysis of red wines

Next, we analyzed a red wine sample (1:5 dilution, *v/v*) in n = 9 replicate measurements with the optimized conditions as described before. Using both electrospray modes, ESI(-) and ESI(+), in total 4400 different features were found in all nine replicate measurements but not in blank samples, respectively (Fig. 3A-C). Only 255 features, having the same neutral monoisotopic mass (± 10 ppm) and retention time (± 0.3 min) could be detected likewise in

both ionization modes (Fig. 3C). 147 of the features found in both ionization modes could be separated by RP chromatography compared to 101 features separated by HILIC. Overall, in ESI(+) mode we could detect approx. twice the number of features as compared to ESI(-). However, the number of features was 1.7 and 2.4 times higher in ESI(+) after HILIC and RP separation, respectively. The different factors could arise from the type of compounds separated by the two stationary phases and the eluent composition. Here, we used in both electrospray modes addition of 0.1% formic acid to the RP eluent which is known to enhance MS response in positive ionization mode. By comparison, the HILIC separation was performed using an almost neutral eluent composition of 5 mM ammonium formate and ACN.

3.4.1 Precision of the method

The precision of retention times was evaluated by injection of n = 9 replicates of a red wine sample. The relative standard deviation (RSD) was <2% for all 4400 detected features derived from the original retention times (none-aligned retention times). This is in good agreement with other dual-column methods [11, 15]. Additionally, the majority (>98%) of detected features showed a random distribution of retention time values among the nine replicates and none of the retention times showed a relative deviation greater 3% from its mean, respectively (Fig. 3D). In general, features detected during the RP separation revealed slightly lower run-to-run repeatability than those detected during the HILIC separation.

3.4.2 Retention characteristics of the stationary phases

We searched for possible compound classes in FooDB (http://foodb.ca, release: 06/29/2017) to gain more information about the retention behavior of the two columns. Database searches were done only based on the experimental monoisotopic mass (± 10 ppm) and multiple

annotations were allowed. This results in a list of possible compound annotations and the corresponding compound classes. Although this approach does not provide unambiguous identifications, it gives valuable information about the distribution of compound classes between the two columns. Most of the putatively metabolites found in the database belong to compound classes that are known for their relevance in wine (Fig. 4). The majority of compounds separated by the RP column were assigned as phenylpropanoids, polyketides, lipids and lipid-like molecules (68% of database findings) indicating especially a strong selectivity of the RP column for flavonoids. The number of possibly detected flavonoids was slightly higher in positive ionization (Fig. 4B), even though in most studies ESI(-) is the method of choice for the analysis of these type of compounds [22, 23]. On the one hand, it must be noted that we used addition of 0.1% formic acid consistently in both ionization modes for the RP mobile phase which could play a role in the preferred ionization efficiency in ESI(+). On the other hand, however, quite a remarkable number of flavonoids, such as anthocyanins, per se are positively charged.

While rather hydrophobic compounds showed a clear preference for the RP column, HILIC revealed stronger retention selectivity for polar compounds, such as carbohydrates and organic acids (Fig. 4). Interestingly, we observed a clear difference in selectivity of the ionization modes for the number of carbohydrates and organic acids putatively annotated in the database. Negative ionization mode could reveal more carbohydrates while carboxylic acids were preferentially detected by ESI(+). Further subdivision revealed that carboxylic acid derivatives detected in ESI(+) were exclusively annotated as amino acids or peptides. By comparison, around 30% of annotated carboxylic acid derivatives found in ESI(-) were mono-, or oligo-carboxylates (17/56) while the remaining 70% were amino acids and peptides (39/56).

3.5 Application to other food samples

We applied the above-described HILIC-RP tandem method to the non-targeted analysis of additional food samples. In this proof-of-concept test, we measured a beef meat extract and a diluted Italian espresso (coffee extract). After chromatographic separation, food components were recorded by MS in ESI(+) mode. Both samples showed a comparable number of recorded features (Supporting Information). The total number of detected features was 1633 and 1797 found in the meat extract and brewed coffee, respectively. Similar to the results we obtained for the red wine sample, in both samples approx. 50% of the features were recorded after HILIC separation indicating an equal distribution of sample compounds. Hence, the method presented here is not limited to the analysis of wine samples. It rather can be used as a routine platform in the non-target screening of different food samples.

The same database search as described above revealed that most of the features detected in the meat extract could be attributed to lipid- and organic acid derivatives, including amino acids and peptides (Fig. S1). Although fatty acids were partially removed in the sample preparation, more than 70% of database findings of those compounds retained on the RP column were lipid-type molecules. Main compound classes found for coffee metabolites were lipid-like molecules, benzenoids, phenylpropanoids, phenylketides, and heterocyclic compounds (*e.g.* furans, pyrans and pyrazines) (Fig. S1).

4 Concluding remarks

The presented HILIC-RP tandem coupling allows sensitive and precise analysis of food metabolites covering a large polarity range in a single chromatographic run. We showed that the combination of two columns with high orthogonality (HILIC and RP) increases the

polarity range covered from highly polar (*e.g.* carbohydrates, oligo-carboxylic acids) to unpolar compounds (*e.g.* flavonoids) which is of particular importance in comprehensive non-targeted studies. Analysis of a red wine sample revealed more than 4000 features detected by high-resolution MS. Although we mainly used a red wine sample for method development and validation, the potential of this method in the application to other types of food samples has been proven. Moreover, there is no reason to be restricted to foods. We believe that this method is valuable for the analysis of small molecules (metabolites) in a wide range of different sample matrices.

Dual-column tandem chromatography does not require complicated instrumentation and software setups such as in comprehensive 2D-LC. Compared to serial couplings individual gradients for each separation column can be used independently. In theory, there is no limitation in the types of columns, which are connected in the system. When the aim is to measure as many compounds as possible in a single run, columns of highest possible orthogonality (complementary columns) should be favored. Nevertheless, combination of less complementary columns could have also specific advantages. For example, coupling of two RP columns with different surface chemistries (*e.g.* Phenyl/C18 or C8/C18) could enhance the total peak capacity, selectivity and separation efficiency specifically for hydrophobic compound classes (*e.g.* lipids and lipid-type molecules) compared to single column LC. Depending on the sample extraction, in some cases it could be useful to change the order of the two columns. Sample extracts containing high amounts of organic solvent (*e.g.* after protein precipitation) could benefit from a reversed column order and a hydrophilic trap column. Optimization regarding high-throughput and run-time reduction was not the aim of this study. Further method development is needed to reduce sections in the chromatogram of poor peak capacity. Additionally, the use of columns with smaller particles (UPLC) may further reduce the overall run time.

Conflict of interest statement

The authors declare no conflict of interest.

5 References

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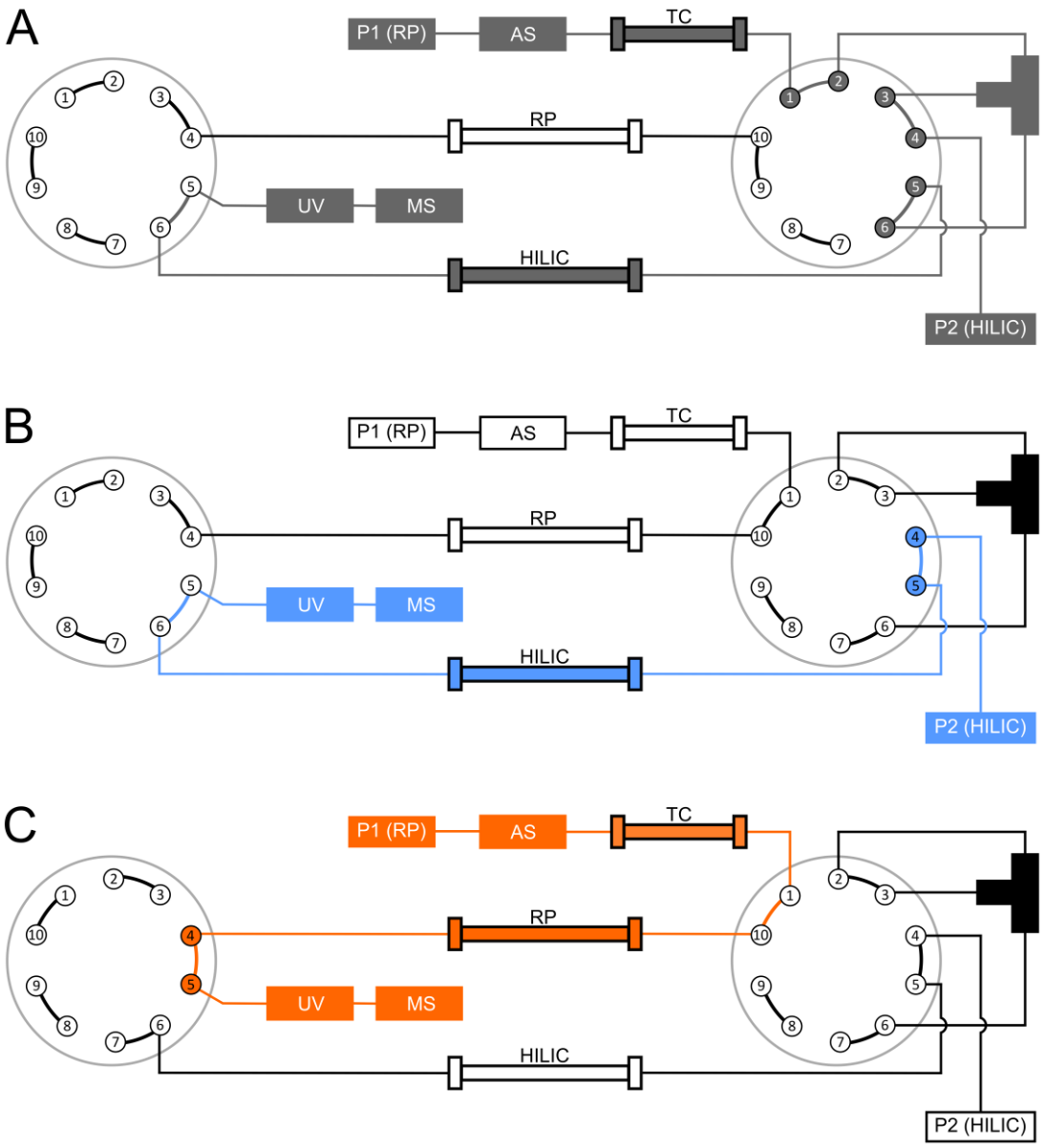

Figure 1 | Configuration of the tandem HILIC-RP system. (A) Pre-fractionation of the sample in a first loading (trapping) step (0-5 min) into hydrophobic and hydrophilic compounds. Addition of high organic mobile phase via pump 2 and a T-piece ensures retention of hydrophilic compounds on the HILIC column. (B) Independent gradient elution of hydrophilic components retained on the HILIC column (5-25 min). (C) Independent gradient elution of hydrophobic components trapped on the RP column (25-45 min).

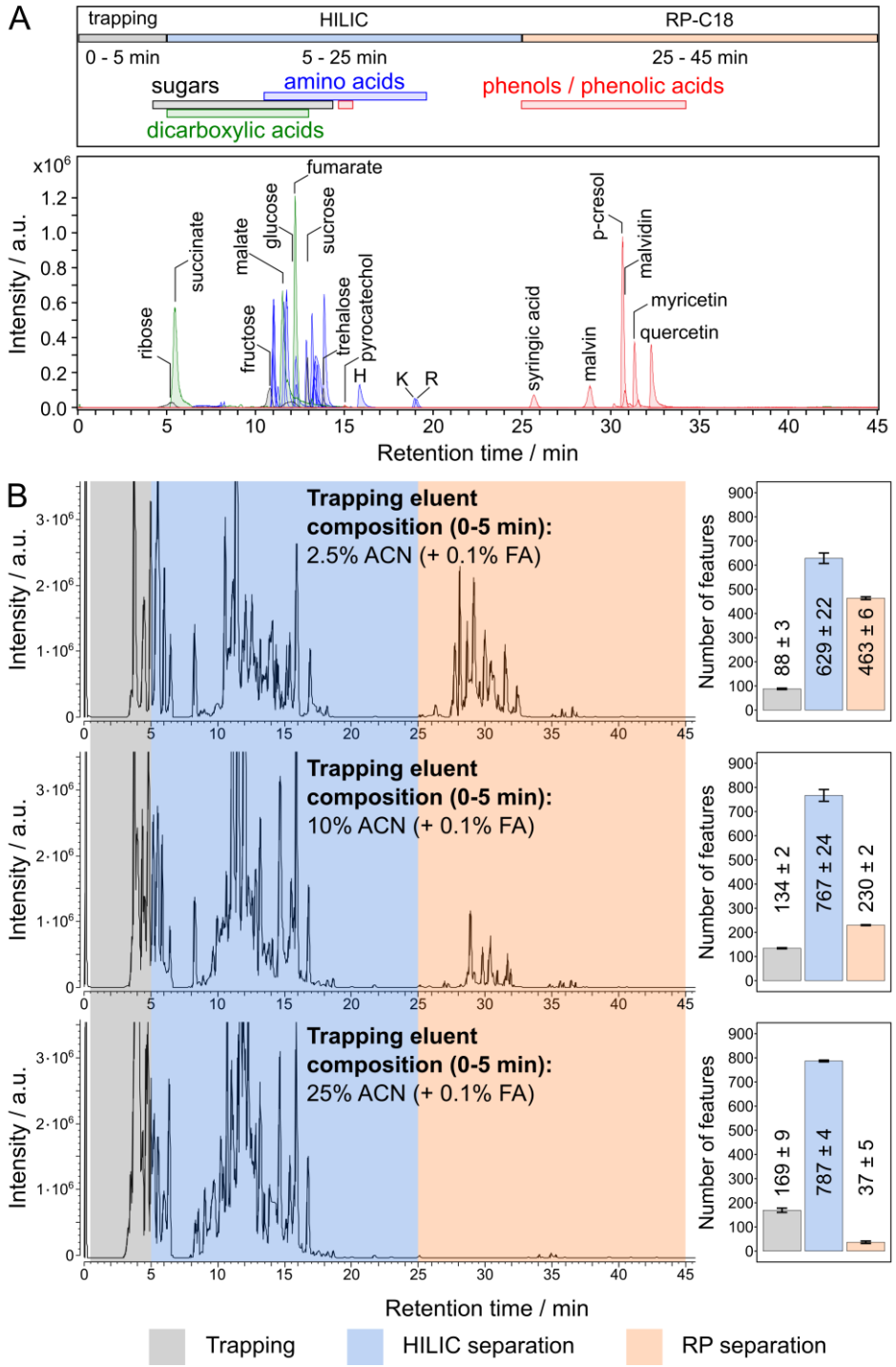

Figure 2 | (A) Analysis of 34 food relevant reference standards including amino acids, mono- and disaccharides, dicarboxylic acids, and phenols. (B) Influence of the acetonitrile content in the eluent composition during the loading step (0-5 min) on the distribution of red wine components onto the HILIC or RP stationary phase. Bar charts illustrate the number of detected features (S/N > 3) in the three sections of the chromatogram, respectively. Error bars indicate the standard deviation of the mean (n = 3).

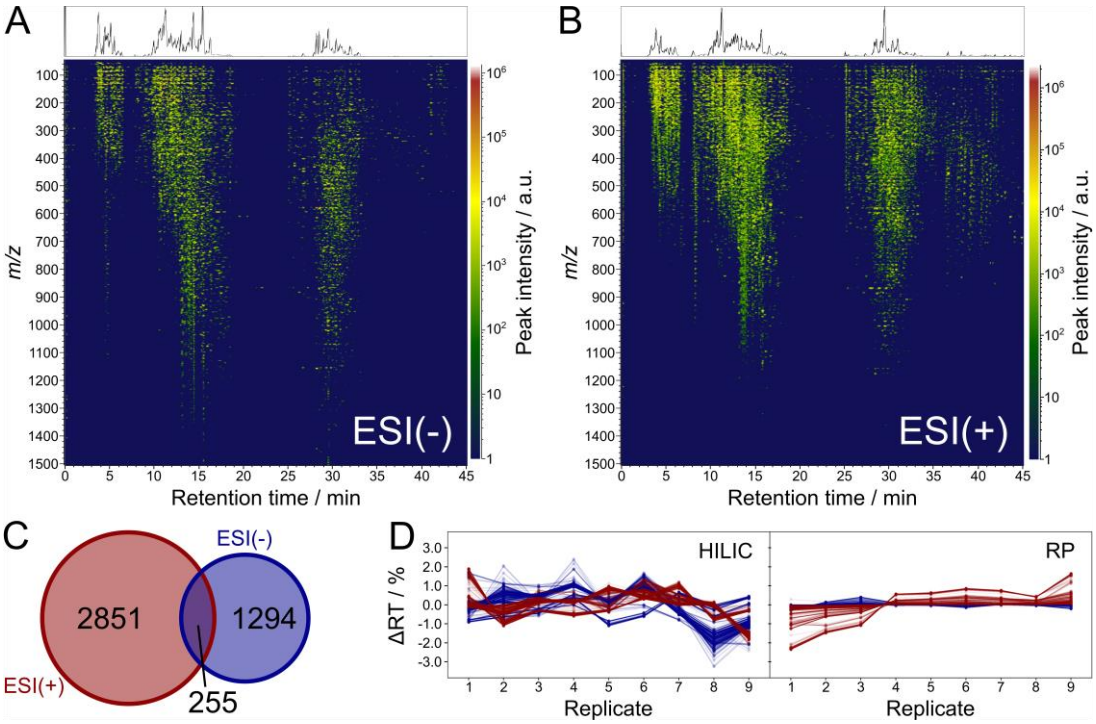

Figure 3 | Non-target analysis of a red wine sample by tandem HILIC-RP chromatography. (A-B) Retention time versus *m/z*-value plots of processed results obtained in electrospray positive (ESI(+)) and negative (ESI(-)) mode, respectively. Each dot represents an analytical signal (feature) colored according to the observed peak intensity. (C) Comparison of detected features in ESI(+) and ESI(-) mode. (D) Relative deviation of the retention times of all detected features from the mean for nine replicate measurements detected during HILIC (left) and RP separation (right); ESI(+) = red; ESI(-) = blue.

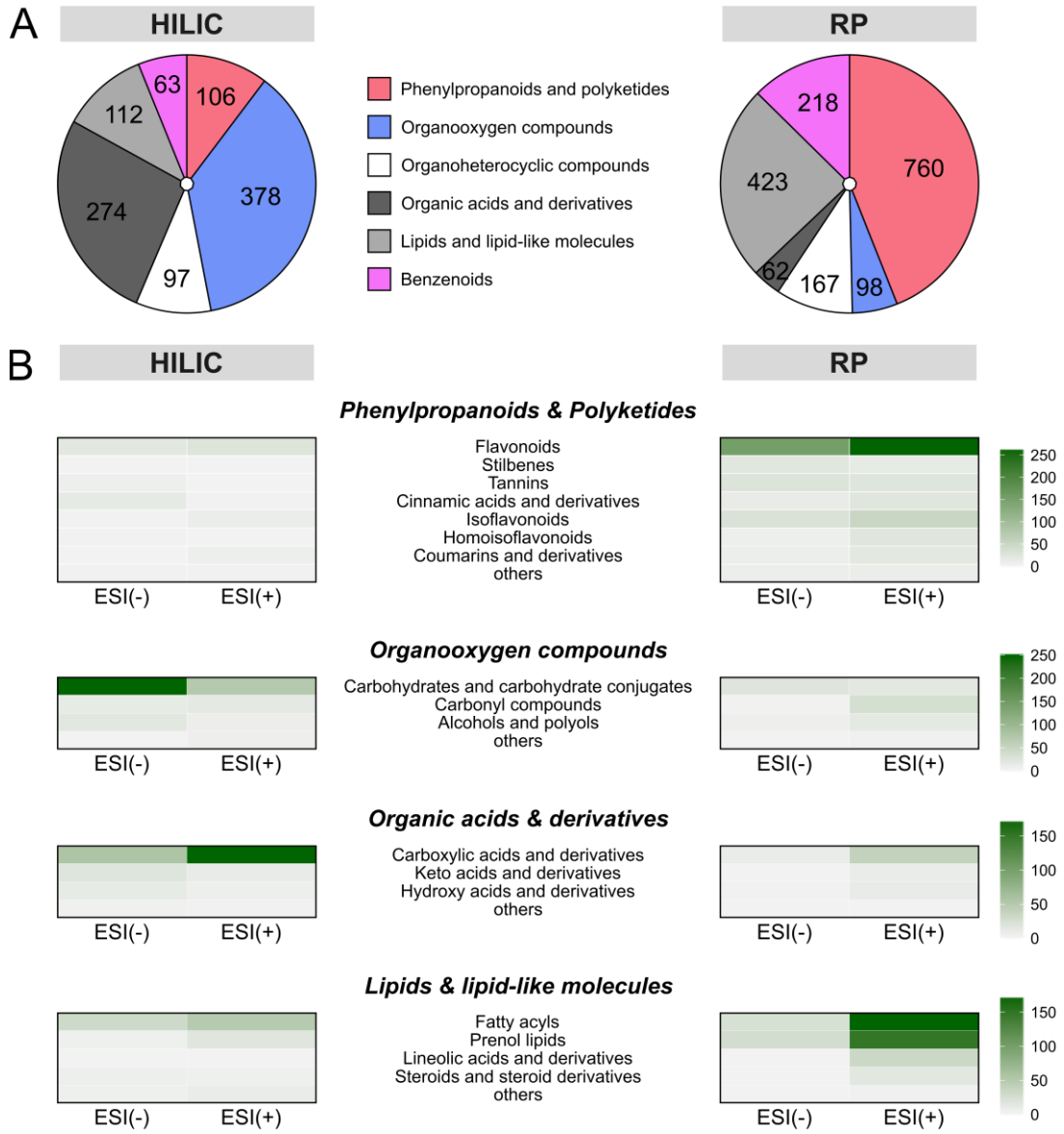

Figure 4 | Selectivity of the two analytical columns used in the tandem LC system and selectivity of ionization modes for food relevant compound classes. (A) Pie charts illustrating the preferred selectivity of the HILIC and RP column. (B) Sub classification of major compound classes.

Table 1 | Chromatographic conditions of the tandem HILIC-RP coupling.

			Pump 1 (P1)		Pump 2 (P2)	Valve position
	Time (min)	%B	Flow (µL/min)	%B	Flow (µL/min)	Left | right
Trap.	0.0	2.5	50	100.0	350	1_2 | 1_2
	5.0	↓	↓	↓	350	1_2 | 1_2
HILIC	5.1	↓	50	100.0	400	1_2 | 10_1
	18.0	↓	↓	40.0	↓	↓
	24.0	↓	↓	40.0	400	↓
	24.9	↓	400	↓	↓	1_2 | 10_1
RP	25.0	↓	↓	↓	↓	10_1 | 10_1
	26.0	2.5	↓	90.0	200	↓
	40.0	100.0	↓	↓	↓	↓
	45.0	100.0	↓	↓	↓	↓
Equil.	48.0	2.5	↓	↓	↓	↓
	60.0	2.5	400	90.0	200	10_1 | 10_1