Date: 12 Oct 2019

To: "Piera Di Martino" piera.dimartino@unicam.it

From: "Journal of Chromatography A" eesserver@eesmail.elsevier.com

Subject: Manuscript JCA-19-1310R1

Reply To: "Journal of Chromatography A" chrom-eo@elsevier.com
Manuscript number: JCA-19-1310R1
Manuscript title: Quantification of phenolic compounds in different types of crafts beers, worts, starting and spent ingredients by liquid chromatography-tandem mass spectrometry
Author(s): Manuela Cortese; Maria Rosa Gigliobianco; Dolores Vargas; Gianni Sagratini; Roberta Censi; Piera Di Martino, Prof
Submitted to: Journal of Chromatography A

Dear Professor Di Martino,

I am pleased to inform you about the acceptance of the manuscript entitled:

"Quantification of phenolic compounds in different types of crafts beers, worts, starting and spent ingredients by liquid chromatography-tandem mass spectrometry"

Your paper will soon be passed to the production department for further handling. Then, you will receive further information regarding page proofs, transfer of copyright, etc.

With kind regards

Colin Poole
Editor
Journal of Chromatography A

Comments (if any):

_____

Quantification of phenolic compounds in different types of crafts beers, worts, starting and spent ingredients by liquid chromatography-tandem mass spectrometry

Manuela Cortese[§], Maria Rosa Gigliobianco[§], Dolores Vargas Peregrina, Gianni Sagratini, Roberta Censi, Piera Di Martino*,

School of Pharmacy, University of Camerino, Italy

§ Authors contributed equally

***** Author for correspondence

Piera Di Martino

University of Camerino, School of Pharmacy,

Via S. Agostino, 1

62032 Camerino (Italy)

Tel.: +39 0737 402215

Fax: +39 0737 637345

e-mail: piera.dimartino@unicam.it

Abstract

Phenolic compoundsphenolic compoundsphenolic compoundspresent in several kind of craft beers, corresponding worts, ingredients and spent products was performed by LC-MS/MS. The dilution 1:2 of the sample with the mobile phase gave the best results, offering a very fast and simple method to reduce the matrix effect. A validated method was applied to six different types of craft beers, their worts, starting and spent products, such as barley malts and barley husks, starting hops and spent hops, and finally, starting yeast and spent yeasts to quantify the selected phenolic compounds. The Total Phenol Content (TPC) of barley malts is not negligible and it results almost prevalently due to trans-*p*-coumaric acid, which ranges from 76.4 μg/Kg for Mais to 672.6 μg/Kg for Amber. The trans-*p*-coumaric acid is transferred to the worts during the must preparation and is responsible for the not negligible TPC of worts, that was between 131.1 μg/Kg for Ego to 2041.6 μg/Kg for Alter beer. Bitter acids and prenylflavonoids are mainly present in the starting hops (TPC 323.8 μg/Kg and TPC 500.3 for Saaz and Perle hops, respectively). Their concentration strongly decreases in the spent hops where the TPC ranges between 8.0 μg/Kg for Triple Malt to 24.4 μg/Kg for Alter, suggesting that they are transferred to the intermediate of production. Phenolic compounds, originally present in the starting barley malts and hops, are limitedly present into the final beers, and their TPC ranges approximately from 65.6 μg/Kg for Fiat lux to 105.3 μg/Kg for Alter. Actually, most phenolic compounds are absorbed into the yeast added for the fermentation, as it is clearly evident from the observation that spent yeasts contain a higher phenolic compounds amount with the respect to the starting yeast, and several phenolic compounds, in particular those coming from hops, are originally absent into the yeast and are only present in the spent ones.

Keywords: HPLC-ESI-MS/MS, matrix effect, method validation, craft beers, beer by-products, phenolic compounds.

1. Introduction

Beer is not only one of the oldest alcoholic beverage, but also the most produced and consumed in the world [1]. It is a very complex beverage and it is composed by water (more than 90%), alcohols resulting from the fermentation process (approximately ethanol 5.0% and glycerol 0.5%), carbohydrates deriving from barley malt (mostly non-fermentable dextrins and α-glucans), minerals (cations such as magnesium, potassium, sodium and calcium; and anions like chloride, sulfate, nitrate and phosphate), water soluble vitamins of the B-group (folate, riboflavin, panthotenic acid, pyridoxine, thiamin and niacin), and phenolic compounds [2], 70-80% of which came from barley malt and the remaining part from hops, according to the literature [3]. Thanks to the presence of these bioactive compounds, it has been widely proved that commercial beers have important antioxidant activity [4–9]. The European Prospective Investigation into Cancer and Nutrition cohort study reported that beer is the main contributor to hydroxybenzoic acid intake and it is a good source of phenolic compounds [10].

Using liquid chromatography coupled with an electrospray ionization source hybrid linear ion trap quadrupole Orbitrap, for the first time Quifer-Rada et al. [11] performed a comprehensive characterization of four types of commercial beer, identifying 47 antioxidant compounds, belonging to phenolic acids, flavonoids, bitter acids, prenylflavonoids, alkylmethoxyphenols, and indole-based compounds. More recently, Cheiran et al. [12] increased the number of identified molecules to 57 phenolic species and 11 nitrogenous compounds in craft beers. Even if extensive qualitative evaluation of phenolic compounds was reported in literature, a comprehensive quantitative approach is missing. In our study, we monitored and quantified phenolic compounds belonging to the most compounds contained in the beer: phenolic acids (gallic acid, chlorogenic acid (3-CQA), 4-O-Caffeoylquinic acid (4-CQA), 4-hydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid, *p*-coumaric acid, *t*-ferulic acid, sinapic acid), flavonoids ((+)-catechin, (-)-epicatechin, quercetin), bitter acids (humulone and cohumulone, lupulone and colupulone), prenylflavonoids (xanthohumol,

isoxanthohumol and 8-prenylnaringenin). The quantification of some of these species was performed combining the chromatographic separation with different detectors, such as electrochemical [6], spectrophotometric [13], low resolution mass spectrometry in tandem mass mode (HPLC-MS/MS) [12]. Unfortunately, even using a specific and sensitive technique like HPLC-MS/MS, the presence of interferences, belonging from the beer complex matrix, can strongly affect the quantification of phenolic compounds. Polar compounds like carbohydrates and vitamins can co-elute with the target analytes and compete with them for charge acquisition on the droplet surface during the ionization process occurring in the interface system (Ionization Source) [14]. To obtain accurate results, an extensive matrix effect evaluation is needed. In literature, different strategies are reported to overcome the matrix effect (ME) [14].

In this study, craft beer were analyzed instead of commercial ones by considering that the popularity of niche beers has increased in recent years and the market is progressively shifting from mass-produced lager brands to the richer flavors, quirky ingredients, and striking aesthetics of craft beers, which are characterized by a unique aroma and taste [15]. In particular, in recent years in Italy, it has been possible to assist to a remarkable increment in the craft beers production [16]. In addition, several studies are reported in literature on characterisation and quantitation of phenolic compounds in commercial beers, but only few [12,17–19] were carried out on craft beers, and how their production process affects polyphenol content and antioxidant activity.

Moreover, brewery by-products should keep antioxidants in different amount according to the type of beers and brewery process, but these aspects have never been previously investigated. Thus, brewery by-products could be reintroduced in a circular economy as a source of phenolic compounds to be included in health products, such as supplements, nutraceuticals, functional foods, and cosmetics.

Starting from these premises, the first aim of this work was to explore how each single action useful to overcome Matrix Effect (ME) influences the chromatographic profile and the quantification of phenolic compounds in a complex matrix like craft beer, and to propose a methodological approach

for a fast identification of the best and the easiest strategy to minimize ME in HPLC-MS/MS analysis. This study was planned and performed on one type model hand craft beer, the Ego Alter beer, arbitrarily selected as model beer, and the method was completely validated.

The second aim of this work was to provide, for the first time at our knowledge, an accurate and comprehensive quantification of 20 selected phenolic compounds in six different types of craft beers, in the corresponding worts, in the starting and spent products, such as barley malts and barley husks, starting hops and spent hops, and finally starting yeast and spent yeasts, by using a validated HPLC-MS/MS method. Extractions were performed in water in view of a sustainable exploitation of beer by-products.

2. Experimental

2.1 Chemicals and materials

The craft beers were kindly supplied by "Birrificio artigianale Collesi" from Apecchio (PU, Italy) and they belong to the "Linea Imperiale". Six different types of craft beers were analysed: "Ego", "Alter" and "Triplo Malto" are lager beers, "Ubi" is a red beer, "Fiat Lux" is a amber beer, and "Maior" is a black beer. They differ for the starting barley malt (Mais, Special Black, Pilsen, Munich, Amber) and hop types (Peerle and Saaz), while the yeast type (Saccharomyces Cerevisiae) was always the same. Other variables are related to the process temperature and time. Details about the receipt and the brewery process could not be provided because strictly confidential. During the study, together with every craft beers, the starting barley malts, hops, yeast, worts, spent burley husks, hops and yeast were analysed.

The analytical standards for the HPLC-MS/MS (gallic acid, vanillic acid (97% HPLC), *p*-coumaric acid (≥98%HPLC), *trans*-ferulic acid (99%), sinapic acid (≥98%), caffeic acid (≥98% HPLC), siringic acid, 4-hydroxibenzoic acid, (+)-catechin, hydrate quercetin (≥95%), 3-caffeoylquinic acid (3-CQA), xantohumol, isoxantohumol, 8-prenylnarigenin were purchased from Sigma-Aldrich (Stenheim, Germany). Cohumulone, colupulone, lupulone, colupulone were purchased as the

International calibration Extract 4 (cohumulone 10.98%, colupulone 13.02%, lupulone 13.52%, colupulone 31.60%) from Labor Veritas AG (Postfach, Zurich, Switzerland).

Methanol, formic acid, and hydrochloric acid (35%) for LC/MS were purchased from Carlo Erba Reagents (Cornaredo, MI, Italy). Ethyl acetate for the liquid-liquid extraction was purchased from Merck (Darmstadt, Germany).

The ultrapure water was produced from the Millipore system (Millipore Sigma, Darmstadt, Germany), and filtered with a 0.20 µm Sartolon polyamide filter (Sartorius Stedim Biotech, Göttingen, Germany).

The Solid Phase Extraction (SPE) cartridges (Chromabond PS-OH-, 3 ml/500 mg) for the sample clean up were purchased from Macherey-Nagel (Duren, Germania), while the Simplified Liquid Extraction (SLE) cartridges (Novum 12 cc Tube) were purchased from Phenomenex (Castel Maggiore, BO, Italy).

2.2 Sample preparation

Prior to the analyses, the starting materials (barley malt, hops, and yeast), beers, worts, and waste (barley husks, hop, and yeast) were subjected to different processes according to their physical state, which could be dried solid, humid solid, or turbid liquid.

Humid solids and liquids such as spent materials, worts, and beers were first subjected to lyophilisation at a temperature of -50 °C and a pressure of 0.03 millibar (FreeZone 1 Liter Benchtop Series 77400 freeze-dryer, LABCONCO, Kansans City, MO, USA). Dried solids such as starting barley malts, hops and yeast were subjected to milling in a cutter miller.

Next, all the samples were subjected to extractions. One gram of dried sample was carefully weighed and dispersed in 100 ml of ultrapure water. Ultrapure water was used as extraction solvent according to spectrophotometric preliminary results (unpublished data) and in view of further exploitation of extracts for the preparation of sustainable products such as supplements, nutraceuticals, functional foods, and cosmetics. The resulting liquid was placed in Erlenmeyer flask

hermetically closed. Samples were magnetically stirred for 24 hours at room temperature, then centrifuged at 90000 g at 20 °C for 10 minutes to remove undissolved particles (Zetalab CNZ-140H-E, Padova, Italy). Samples were lyophilized and stored at -20 °C in 50 ml polyethylene vials with screw cap (BD Falcon ™, BD Biosciences, Bedford, MA, USA) in order to ensure optimal storage conditions. The lyophilized solids were thus reconstituted by dissolution in ultrapure water for further uses.

2.3 ME minimization

2.3.1 Dilution approach

A volume of 1 ml of solution obtained by dissolving lyophilized beer in ultrapure water was diluted with water/formic acid 0.1% at the following ratios: 1:2, 1:5 e 1:10 [20–22]. The diluted sample was filtered with a 0.45 µm filter and then with a 0.20 µm filter (Sartorius Stedim Biotech, Göttingen, Germany). The samples were immediately analysed after preparation. The 1:2 dilution was the method applied for the analysis of all the samples under study. Initially, it was applied only to Ego beer for the method validation and the evaluation of ME.

2.3.2 Clean up with Solid Phase Extraction (SPE)

A volume of 1 ml of solution obtained by dissolving lyophilized beer in ultrapure water was purified by the Solid Phase Extraction (SPE) according to Quifer-Rada et al. [11] to reduce the ME. Briefly, the SPE cartridge was activated with 5 ml of methanol and then conditioned with 5 ml of sodium acetate 50 mM at a pH of 7.0. A volume of 1ml of solution acidified with 34 µl of HCl at 35%, was loaded into the cartridge. Then the cartridge was rinsed with 2 ml of a solution of sodium acetate 50 mM at pH 7.0 containing 5% of methanol, Phenolic compounds were eluted with 2 ml of methanol with 2% formic acid. The eluted solution was recovered in a Erlenmeyer flask, dried under vacuum, and dissolved again in ultrapure water until a final dilution of 1:2. The sample was filtered with a 0.45 µm filter and then with a 0.2 µm filter (Sartorius Stedim Biotech, Göttingen,

Germany) before the injection in the HPLC apparatus. This method was only considered for the Ego Lager beer selected as model beer.

2.3.3 Clean up with the Simplified Liquid Extraction (SLE)

The sample was purified by the Simplified Liquid Extraction (SLE). 1 ml of sample beer was diluted with 1:2 water/formic acid at 0.1%. After 5 minutes necessary for the sample adsorption into the cartridge, the analytes were diluted with 20 ml of ethyl acetate. The solution drop occurred by gravity. The cartridge was then dried under vacuum. The elute was desiccated under reduced pressure. The residue was collected with 2 ml of water/formic acid 0.1% and methanol (70:30), by using the initial same conditions of the mobile phase of the chromatographic process. The total dilution was of 1:2. The diluted sample was filtered with a 0.45 µm filter and then with a 0.2 µm filter (Sartorius Stedim Biotech, Göttingen, Germany) before the injection in the HPLC apparatus. This method was only considered for the Ego Lager beer selected as model beer.

2.4 HPLC-API-MS/MS method

The HPLC-API-MS/MS was carried out by a HPLC apparatus (HPLC Agilent 1290 Infinity, Agilent Techonologies, Santa Clara, California, USA) coupled with a triple quadrupole mass spectrometer (MS-QQQ) (Series 6420, Agilent Techonologis Santa Clara, California, USA) equipped with a ESI (electrospray ionization) source in negative polarity. The working conditions of the ESI source were the following: gas temperature 300 °C, gas flow 12 l/min, nebulizer pressure 45 psi, the capillary voltage 4000 V, and the chamber current 3 µA.. Beer samples were analyzed in Multiple Reaction Monitoring (MRM) method, that permitted to increase the analysis specificity.

The liquid chromatography was carried out by the HPLC equipped with binary pump and auto-sampler. A constant temperature of 30 °C permits to keep constant the retention time of each analyte, an important parameter when using the acquisition window. The chromatographic separation was carried out in presence of a Synergi Polar 150 x 4.6 mm column with 4 µm particle diameter (Phenomenex, Castel Maggiore (BO), Italy). The analysis was carried out in presence of a

gradient elution with a mobile phase of water /0.1% formic acid (solvent A) and methanol/0.1% formic acid (solvent B) at a constant flow of 0.7 ml/min. The optimized gradient is not linear: 0 min, 30% B; 0-5 min, 30% B; 5-10 min, 40% B; 10-20 min, 80% B; 20-22 min, 80% B, 22-27 min, 30% B; 27-35 min, 30% B. The injection volume was 10 µl. After validation and evaluation of ME, HPLC-ESI-MS/MS was selected as the best method and applied for the analysis of all the samples.

2.5 Method validation

The method was validated for linearity, accuracy, precision, sensitivity (LOD and LOQ) according to the Food and Drug Administration Guidelines (FDA) [23] .

The linearity was determined by considering the linear regression coefficient (R^2) of a strength line constructed starting from standard solutions of known concentration in the calibration interval of LOQ of each analyte and 1000 µg/l.The accuracy was assessed by the injection of a 250 µg/l standard solution for three times, the standard deviation percentage of the replicated measures were calculated and reported in Table 1.

The precision was evaluated from the inter day repeatability. It was evaluated for a 250 µg/l standard solution. This standard solution was injected during four different days and the standard deviation from a mean value were calculated and reported in Table 1.

The sensitivity was determined according to the limit of quantification (LOD) and limit of detection (LOQ).

The LOD value were defined as the concentration at which the ratio between the peak height of each analyte (S) and the noise (N) is equal to 3.

Instead for the LOQ values the S/N ratio must be equal to 10.

Another parameter that must be taken in consideration during the validation process is the ME, that evaluates the influence of a complex matrix in the quantification of every phenolic compound contained in the sample.

The Matrix Effect (ME) can be calculated according to the equation (1):

$$ME = \left| \frac{(\text{Area of peak in the fortified beer-Area of the peak in beer})}{\text{Area of the standard solution}} * 100 \right| \quad \textbf{equation (1)}$$

3. Results and Discussion

3.1 Brewing process of craft beers

Figure 1 illustrates the brewing process of craft beers used for this work. The craft beer brewing process begins with the mixing of barley malts and water in appropriate proportions. Five different barley malts can be used and mixed together according to different undisclosed recipes: Mais, Special Black, Pilsen, Munich, Amber. Water and barley malts are heated at a temperature of 70 °C for 90 minutes and the resulting wort is filtered to remove the spent barley malt. In the next step, two different hops, Perle and Saaz, can be used in different proportions. The hops are added to the filtered wort and boiled at 100 °C for 90 minutes, after which the spent hops are removed by centrifugation. The subsequent step is fermentation, when *Saccharomices Cerevisiae* yeast is added and heated at 20-22 °C for 90 minutes to convert the sugars into alcohol. The spent yeast is then removed by centrifugation, the resulting beer is bottled and, after a variable period of maturation of 20-30 days, is ready for consumption.

To summarise, the brewing ingredients are water, barley malts, hops, and yeast. The intermediate product is the wort, and the final one is the matured beer. The spent materials are barley malt, hops and yeast.

3.2 HPLC-MS/MS conditions

In this study, all the phenolic compounds were analysed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) using negative ion mode. Each monitored transition was optimized using the corresponding standard to get the maximum response from the instrument using the deprotonated $[M-H]^-$ species as the precursor ion. Five acquisition windows were created in order to minimize the number of transitions monitored at the same time, and the dwell time was maximized

taking in consideration the ionization capability of each analyte (lower is the ionization capability, higher value of the dwell time was set-up).

In our experience and accordingly to literature, the Synergi Polar C18 column was an effective column for the separation of polar bioactive compounds such us phenolic compounds [24-25]. Regarding the mobile phase, different organic modifiers (methanol and acetonitrile) and additives (formic acid, acetic acid) were assessed and their performance were evaluated on the base of the peak shape and relative abundance of analytes. The best conditions were achieved using water and methanol both containing 0,1% (v/v) of formic acid. The elution gradient was optimized in order to obtain the best separation of all analytes.

3.3 Method validation

Method for HPLC-ESI-MS/MS analysis was validated for linearity, accuracy, precision, sensitivity, and ME for Ego beer. The linearity range was assessed using different concentrations of a standard solution: the lower limit of the calibration range was the limit of quantification (LOQ) detected for each analyte, while the upper concentration level was 1000 µg/l for all the analytes. From the data, a strength line was constructed and linearity was determined through the linear regression coefficient (R^2) for values between 0.9905 and 0.9998. Accuracy was evaluated performing triplicate analyses of a standard solution of 250 µg/l. The accuracy values were expressed as standard deviations percentage from 0.7 to 7.3% (Table 1). Method precision was determined from a standard solution of 250.0 µg/l: the standard deviation was calculated from quadruplicated analyses to evaluate the instrument responses during different days. Standard deviations percentage ranged from 0.8 to 10.2% (Table 1). Sensitivity was assessed through the Limit of Detection (LOD) and Limit of Quantitation (LOQ): standard solutions of increasing concentrations (ratio signal to noise is between 3 and 10) were injected. LOD varied between 1 and 150 µg/l, while LOQ values varied from 4 to 500 µg/l respectively for 8-prenylnaringenin and vanillic acid. Matrix Effect (ME) resulted particularly high, thus the study was separately considered in depth for the minimization of

ME (see following paragraph 3.4). For accurate results the ME was determined for each analyte in each craft beer and corresponding by-products (Table 2).

3.4 ME minimization

Several techniques were applied to minimize ME in craft beer, within the scope to finally select the most easy and quick method for routinely applications [14]. The techniques considered for the present study were dilution, or different sample clean-up procedures (SPE and SLE). According to literature and our experience, the best ionization polarity for phenolic compounds is the negative ion mode. lower

Different dilution factors were selected for this study. The optimal result was evaluated with respect of reduction of ME, without be detrimental on sensitivity. The dilutions water/0.1% formic acid were 1:2, 1:5, and 1:10. The following ME values were obtained for the different dilutions: for 1:2 ME was between 35.7% and 137.1%; for 1:5 ME was between 34.4% and 83.2%; for 1:10 ME was between 44.2 and 64.7%. By comparing the three dilution factors, neglecting advantages were observed in term of ME reduction, while increasing dilution factor; meanwhile, there is a strong reduction in sensitivity. The best compromise between the ME reduction and sensitivity was guaranteed for a 1:2 dilution (Figure 2). In particular, it is possible to highlight that the ME values are higher for the analytes in the first part of the chromatogram (gallic acid and catechin) for all dilution factors. One possible explanation belong to the co-elution of the polar analytes with some polar interferences present in the matrix, such as salts, sugars, and carbohydrates. These species strongly compete for the charge and the ion formation in the liquid phase, even at low concentration, when an ESI source is used. This induces a suppression or an increase in the ion formation, leading to higher ME values. For those less polar, as for quercetin, the ME decreased. This mechanism is well described in literature by Trufelli et al. [14], where they reported that most polar and small molecular weight compounds are more prone to ME.

Within scope to minimize ME, the performing of clean up step was then evaluated. In particular, the use of the SPE and SLE techniques was taken in consideration. For the clean- up with SPE, we referred to Quifer-Rada et al. optimized procedure [11], adjusting the final volume of the sample to a dilution factor of 1:2, in order to make a reliable comparison with the ME values obtained only by dilution. The values obtained from SPE are higher than those recovered after dilution, being in the range of 102.5% for the gallic acid to 163.4% for quercetin (Figure 2).

Also in this case, results can be explained in term of polarity of the compounds associated to the clean-up technique used. Actually, ME is rather low for compounds that elute in the first part of the chromatogram, compared to those eluting in the second part. The SPE cartridge can efficaciously eliminate the polar interferences, but it is less effective with respect to less polar interferences, that may be even concentrated. Consequently, there are less polar interferences than can influence the ME for substances such as the gallic acid, and higher amount of interference for substances such as quercetin.

The last technique used to reduce the ME is based on the clean up by SLE. This is an extraction method where the stationary phase of the cartridge behaves like an absorbent without any chemical interaction with analytes, miming a liquid-liquid extraction. By this technique, the ME is constantly reduced for all the analytes (Figure 2), and the ME% varied between 70.4% and 91.8%. Surprisingly, there was a heavy reduction of recovery of chlorogenic acids, even by increasing the elution solvent amount in the SLE procedure. Thus, this technique cannot be considered applicable in our study, even if it seems effective in the ME minimization.

A final comparison was carried out among the following techniques: 1:2 dilution with water/0.1% formic acid, clean up with SPE and clean up with SLE (Figure 2).

By comparing the ME% values obtained for any specific phenolic compound with these techniques, the dilution was revealed as the best technique, because the ME% is the lowest one for the most phenolic compounds. Consequently, this technique was that of choice for the study and it is applied to all the other samples.

The ME% was thus calculated for all the beers and results are given in Figure 3. Each sample revealed a peculiar MEs profile, that underlines the necessity of the evaluation of the ME for each type of beer in order to obtain accurate results. Generally, as expected from previous results, the highest values were obtained for more polar phenolic compounds (gallic acid and chatechin) for any beer sample, while it decreased moving toward less polar one. In general, the Amber and Triplo Malto beers are those more affected by the ME, with values of 10.0-118.0% and 13.6-73.8% respectively. Focusing on the most similar beers in terms of brewing process, Ego Lager and Alter Lager beers differ only for the type of malt used. At the same time significant differences can be highlighted between them on MEs. This finding suggests an important role of malt, the most abundant ingredient in beer processing, on the level of MEs occurrence.

3.5 Quantitation of phenolic compounds in different types of samples by 1:2 dilution approach

Phenolic compounds selected for this study are present in different amounts in the various samples, as reported in Tables 3-8.

The first source of phenolic compounds could be the barley malt, which is the first ingredient used during the brewing process together with water [3]. In Table 3, the content in individual phenolic compounds is reported for the various malts used to brew the beers under study. The barley malts (Mais, Special Black, Pilsen, Munich, Amber) were characterized as pure ingredients and not as the specific mixtures actually used to brew every specific beer, and this is because the receipt was confidential and thus we were unable to reproduce the mixture. The Total Phenol Content (TPC) is not negligible and it approximately ranged from 76.39 μg/Kg for the Mais to 672.6 μg/Kg for the Munich. The content in individual phenolic compounds in the barley malts is relatively low, and almost under the LOQ, with the exception of the trans-*p*-coumaric acid, which is present approximately from 73.0 μg/Kg for the Mais type to 657.2 μg/Kg for the Munich type, and to a very large minor extent, the gallic acid, never higher than 5.8 μg/Kg. The direct comparison of these results with those determined for the barley husks (Table 4) is not possible, because for the

latter the analysis was performed on the mixture of husks actually recovered after the must preparation and not to a single barley malt as was for the former. Nevertheless, by approximately compare the results, it is possible to observe that the polyphenol present in the highest amount in the starting malt, is also the most abundant in the spent products. More in details, the trans-*p*-coumaric acid was the polyphenol found in higher amount in all barley husks, followed by the gallic and 4-hydroxybenozoic acids, while 4-CQA, 3-CQA, epicatechin, vanillic acid, are under the LOQ for all the samples, and sinapic acid, only for some of them. The TPC is generally higher than that of starting barley malts, ranging from 340.0 μg/Kg of Ego to 969.2 μg/Kg of Ubi. It must be noted that the extraction procedures were not the same: barley husks were recovered after the must preparation at 70 °C for 90 minutes, while extractions from barley malts was performed at room temperature for 24 hours. Worts recovered after must preparation and filtration (Table 5) are particularly rich in trans-*p*-coumaric acid (values ranged from 125.27 to 2036.0 μg/Kg), followed by catechin and ferulic acid, that means that must preparation process is able to extract these phenolic compounds from barley malts enriching the worts. Some phenolic compounds are under the LOQ and thus could not be quantified. Among them, 4-CQA and siringic acids were undetectable in all the samples.

After the extraction of barley malt, we obtained by filtration the worts and the corresponding barley husks. The worts were added of hop for the boiling process. Once again, the specific composition in the hop mixture actually used for any specific beer is confidential, and thus we only could analyse the starting hops, Perle and Saaz, as pure ingredients. The starting hop Perle and Saaz are particularly rich in many different phenolic compounds, such as 4-CQA, (+)-catechin, gallic, 4-hydroxybenzoic, caffeic, trans-*p*-coumaric, and trans-ferulic acids for with the content is nearly of the same magnitude (Table 6). It is interesting to observe that, on the contrary, the hop Perle is particularly rich in xanthohumol, humulone, colupulone, lupulone, all substances generally considered characteristics of hops, that are instead very low in the hop Saaz (Table 6). By considering the spent hops, it is possible to observe that the phenolic compounds content strongly

decreased. The TPC of starting hop Perle and Saaz (Table 6) are 500.3 and 323.8, respectively. This values strongly decreased for spent hops, ranging from 8.0 Triplo Malto and 24.4 for Alter. The major contribution to those values is mainly due to gallic acid, trans-ferulic acid, and in few cases to humulone.

The case of the yeast is very interesting (Table 7). The individual polyphenol content of starting yeast is rather low: the total amount is nearly 11 μg/Kg, and the most abundant compounds are the gallic and quercetine. Most phenolic compounds are very low or even under the LOQ. By considering the spent yeast, we can observe an increased amount of individual TPC, which ranged from 12.5 μg/Kg for the Maior to 56.7 μg/Kg for the Triplo Malto. Increased individual phenolic compounds are in particular the colupulone (12.9 μg/Kg for the Triplo Malto), cohumulone and humulone (2.7 and 3.6 μg/Kg for the Ego, respectively), isoxanthohumol (the highest value 3.3 μg/Kg is observed for the Fiat Lux), the trans-ferulic acid is under the LOQ for the starting yeast, and increase up to 11.3 μg/Kg for the Triplo Malto.

Finally, results in polyphenol content for beers are reported in Table 8. The TPC is particularly high for Alter (105.25 μg/Kg), followed by all the others that approximately ranged from 65.6 to 97.8 μg/Kg. The high TPC Alter is almost due to the content in (+)-catechin (34.4 μg/Kg), followed by vanillic, ferulic, 4-hydroxybenzoic acids and isoxantohumol.

Zhao et al. [5] analysed 34 commercial beers quantifying a restricted number of phenolic compounds mostly belonging from malt. Among them the predominant species were gallic and ferulic acids, instead in the craft beers under study the uppermost polyphenol was catechin, followed by ferulic, vanillic and 4-hydroxybenzoic acids (Table 8). The peculiarity is that these compounds were found in negligible amount in the starting malt, and not present at all in hop. Their concentration increases drastically after fermentation. These results can be explained, recognizing an important role of the yeast in the conversion of glycosylated or complex phenolic compounds in the corresponding simple aglycone that can be detected and quantified as the free species.

4. Conclusions

The HPLC-ESI-MS/MS allowed for the determination of 20 phenolic compounds selected for this study in different craft beers, worts, and brewering starting ingredients (barley malt, hop, and yeast) and by-products (barley husk, spent hop, and spent yeast). The method validation and the reduction of ME through a dilution 1:2 was firstly assessed for Ego and thus applied for all the samples, providing satisfying minimization of ME for most of the analytes, through a very simple and fast method. The efforts spent in the minimization of ME by using several approaches, revealed how the preparation step strongly affects the chromatographic profile. A clean-up by SPE cartridges revealed to be effective for the elimination of the more polar interferences and concentrate the target analytes together with apolar interferences eventually present. Then the optimization of the elution step of the SPE clean-up procedure is crucial for a reduction of the amount of apolar interferences, and minimizing ME also in the final portion of the chromatogram. SLE seemed to be the most effective procedure to minimize ME along the entire run, but particular attention has to be paid to the recovery percentages, that can be unacceptable in some cases, like for chlorogenic acids in our study. At least the dilution is a fast, cheap and easy approach for ME minimization, but it can be used only when sensitivity is not crucial. It seemed to be very effective for apolar interferences even at low dilution factor. On the contrary, higher dilution factors were necessary to appreciate a reduction of the ME value, caused by polar interferences, known as more competitive species in the acquisition of the charge on the drop surface inside the ESI source. Beer, similar to an aqueous extract from vegetable sources enriched by a fermentation process which increases the complexity of the matrix, was the best option for general consideration between the preparation procedure used and the corresponding effect on the chromatographic profile in HPLC-ESI-MSMS, observed through the ME values. These general considerations can be applied to every matrix to find easier and quicker solution to minimize ME.

Regarding the application of the validated quantitative analytical method, large differences raised for the different samples in term of TPC and individual polyphenol content. The typical composition of each beer is undoubtedly due to the receipts and to the brewing process. Phenolic compounds present in the beers originate mostly from the barley malt and to a minor extent from the hop. A very large amount of phenolic compounds is still present in the barley husks. The behaviour of the yeast is interesting, which has revealed able to absorb phenolic compounds originating from barley malt and hops in its mass, miming the composition of beer in terms of phenolic compounds. Yeast could be then considered as in interesting source of phenolic compounds from a spent material, as well as spent hops.

Acknowledgements

The authors would like to thank Birrificio Collesi (Apecchio, Italy) for providing samples for brewing process. The authors would also like to thank Ms. Sheila Beatty for editing the English usage of the manuscript, and Mrs Maria Ugili and Sara Pierantoni for their contribution to the experimental work.

The authors also acknowledge receipt of funding from the European Commission of an H2020-MSCA-ITN-2015 award through the ISPIC project (grant number 675743), an H2020-MSCA-RISE-2016 award through the CHARMED project (grant number 734684) and an H2020-MSCA-RISE-2017 award through the CANCER project (grant number 777682).

References

[1] L.C. Nogueira, R.F. do Rio, P.C.B. Lollo, I.M.P.L.V.O. Ferreira, Moderate Alcoholic Beer Consumption: The Effects on the Lipid Profile and Insulin Sensitivity of Adult Men, J. Food Sci. 82 (2017) 1720–1725. doi:10.1111/1750-3841.13746.

[2] V.R. Preedy, Beer in Health and Desease Prevention, Academic Press, 2008.

[3] D. De Keukeleirc, Fundamentals of beer and hop chemistry, Quim. Nova. 23 (2000) 108–112. doi:10.1590/S0100-40422000000100019.

[4] P.A.R. Tafulo, R.B. Queirós, C.M. Delerue-Matos, M.G.F. Sales, Control and comparison of the antioxidant capacity of beers, Food Res. Int. 43 (2010) 1702–1709. doi:10.1016/j.foodres.2010.05.014.

[5] H. Zhao, W. Chen, J. Lu, M. Zhao, Phenolic profiles and antioxidant activities of commercial beers, Food Chem. 119 (2010) 1150–1158. doi:10.1016/j.foodchem.2009.08.028.

[6] A. Piazzon, M. Forte, M. Nardini, Characterization of phenolics content and antioxidant activity of different beer types, J. Agric. Food Chem. 58 (2010) 10677–10683. doi:10.1021/jf101975q.

[7] H. Zhao, W. Fan, J. Dong, J. Lu, J. Chen, L. Shan, Y. Lin, W. Kong, Evaluation of antioxidant activities and total phenolic contents of typical malting barley varieties, Food Chem. 107 (2008) 296–304. doi:10.1016/j.foodchem.2007.08.018.

[8] A. Ghiselli, F. Natella, A. Guidi, L. Montanari, P. Fantozzi, C. Scaccini, Beer increases plasma antioxidant capacity in humans, J Nutr Biochem. 11 (2000) 76–80. doi:10.1016/S0955-2863(99)00077-7.

[9] A. Wei, K. Mura, T. Shibamoto, Antioxidative activity of volatile chemicals extracted from beer, J. Agric. Food Chem. 49 (2001) 4097–4101. doi:10.1021/jf010325e.

[10] R. Zamora-Ros, J. a Rothwell, A. Scalbert, V. Knaze, I. Romieu, N. Slimani, G. Fagherazzi, F. Perquier, M. Touillaud, E. Molina-Montes, J.M. Huerta, A. Barricarte, P. Amiano, V. Menéndez, R. Tumino, M.S. de Magistris, D. Palli, F. Ricceri, S. Sieri, F.L. Crowe, K.-T.

Khaw, N.J. Wareham, V. Grote, K. Li, H. Boeing, J. Förster, A. Trichopoulou, V. Benetou, K. Tsiotas, H.B. Bueno-de-Mesquita, M. Ros, P.H.M. Peeters, A. Tjønneland, J. Halkjær, K. Overvad, U. Ericson, P. Wallström, I. Johansson, R. Landberg, E. Weiderpass, D. Engeset, G. Skeie, P. Wark, E. Riboli, C. a González, Dietary intakes and food sources of phenolic acids in the European Prospective Investigation into Cancer and Nutrition (EPIC) study., Br. J. Nutr. 110 (2013) 1500–11. doi:10.1017/S0007114513000688.

[11] P. Quifer-Rada, A. Vallverdú-Queralt, M. Martínez-Huélamo, G. Chiva-Blanch, O. Jáuregui, R. Estruch, R. Lamuela-Raventós, A comprehensive characterisation of beer polyphenols by high resolution mass spectrometry (LC-ESI-LTQ-Orbitrap-MS), Food Chem. 169 (2015) 336–343. doi:10.1016/j.foodchem.2014.07.154.

[12] K.P. Cheiran, V.P. Raimundo, V. Manfroi, M.J. Anzanello, Simultaneous identification of low-molecular weight phenolic and nitrogen compounds in craft beers by HPLC-ESI-MS / MS, Food Chem. 286 (2019) 113–122. doi:10.1016/j.foodchem.2019.01.198.

[13] O. Galarce-bustos, L. Novoa, J. Pavon-perez, K. Henriquez-aedo, M. Aranda, Chemometric Optimization of QuEChERS Extraction Method for Polyphenol Determination in Beers by Liquid Chromatography with Ultraviolet Detection, Food Anal. Methods. 12 (2019) 448–457. doi:https://doi.org/10.1007/s12161-018-1376-x.

[14] H. Trufelli, P. Palma, G. Famiglini, A. Cappiello, An overview of matrix effects in liquid chromatography–mass spectrometry, Mass Spectrom. Rev. 30 (2011) 491–509. doi:https://doi.org/10.1002/mas.20298.

[15] B. Aquilani, T. Laureti, S. Poponi, L. Secondi, Beer choice and consumption determinants when craft beers are tasted: An exploratory study of consumer preferences, Food Qual. Prefer. 41 (2015) 214–224. doi:10.1016/j.foodqual.2014.12.005.

[16] T. Amoriello, K. Carbone, A. Monteleone, M. Pagano, S. Tarangioli, CRITICITÀ E OPPORTUNITÀ PER LO SVILUPPO SOSTENIBILE, in: 2016. http://sito.entecra.it/portale/public/documenti/brassicola_2016.pdf.

[17] V. Sanna, L. Pretti, Effect of wine barrel ageing or sapa addition on total polyphenol content and antioxidant activities of some Italian craft beers, Int. J. Food Sci. Technol. 50 (2015) 700–707. doi:10.1111/ijfs.12666.

[18] J. Ducruet, P. Rébénaque, S. Diserens, A. Kosińska-Cagnazzo, I. Héritier, W. Andlauer, Amber ale beer enriched with goji berries – The effect on bioactive compound content and sensorial properties, Food Chem. 226 (2017) 109–118. doi:10.1016/j.foodchem.2017.01.047.

[19] P.A. Ulloa, J. Vidal, M.I. Ávila, M. Labbe, S. Cohen, F.N. Salazar, Effect of the Addition of Propolis Extract on Bioactive Compounds and Antioxidant Activity of Craft Beer, 2017 (2017). doi:10.1155/2017/6716053.

[20] H. Stahnke, S. Kittlaus, G. Kempe, C. Hemmerling, L. Alder, The influence of electrospray ion source design on matrix effects, J. Mass Spectrom. 47 (2012) 875–884. doi:10.1002/jms.3047.

[21] M. Suárez, A. Macià, M.P. Romero, M.J. Motilva, Improved liquid chromatography tandem mass spectrometry method for the determination of phenolic compounds in virgin olive oil, J. Chromatogr. A. 1214 (2008) 90–99. doi:10.1016/j.chroma.2008.10.098.

[22] N. Ortega, A. Macià, M.-P. Romero, E. Trullols, J.-R. Morello, N. Anglès, M.-J. Motilva, Rapid Determination of Phenolic Compounds and Alkaloids of Carob Flour by Improved Liquid Chromatography Tandem Mass Spectrometry, J. Agric. Food Chem. 57 (2009) 7239–7244. doi:10.1021/jf901635s.

[23] F. and D.A. FDA, Food and Drug Administration, Guidance for Industry: Bioanalytical method validation., 2001. doi:http://www.labcompliance.de/documents/FDA/FDA-Others/Laboratory/f-507-bioanalytical-4252fnl.pdf.

[24] G. Caprioli, M. Cortese, L. Odello, M. Ricciutelli, G. Sagratini, E. Torregiani, S. Vittori, Importance of Espresso Coffee Machine Parameters on the Extraction of Chlorogenic Acids in a Certified Italian Espresso by Using, 2 (2013) 55–64. doi:10.5539/jfr.v2n3p55.

[25] T.H. Kao, G.Y. Wu, Simultaneous determination of prenylflavonoid and hop bitter acid in

beer lee by HPLC-DAD-MS, Food Chem. 141 (2013) 1218–1226.

doi:10.1016/j.foodchem.2013.04.032.

Table 1

Method validation and HPLC-ESI-MS/MS parameters determined in a large number of phanolic compounds identified in the beer.

Analytes	RT[*1]	Precursor ion (m/z)	Product ion (m/z)	Sensibility[*]		Accuracy[*4]	Precision[*4]
				LOD[*2]	LOQ[*3]		
Gallic Acid	2.68	169	125	19	66	1.9	6.3
Catechin	4.68	289	245	16	54	1.2	3.5
4-CQA	5.07	353	173	29	98	1.8	6.0
3-CQA	5.65	353	191	14	49	1.6	1.5
4-Hydroxybenzoic acid	5.83	137	93	84	278	2.4	2.5
Epicatechin	6.36	289	245	16	54	3.8	4.8
Caffeic Acid	6.84	179	135	28	94	0.7	6.8
Vanillic Acid	7.33	167	152	150	500	7.3	10.2
Syringic Acid	8.97	197	182	60	200	6.7	6.3
trans-p-Coumaric Acid	11.35	163	119	11	36	1.5	4.5
trans-Ferulic Acid	13.51	193	134	115	385	0.7	5.2
Sinapic Acid	14.36	223	208	6	19	1.0	0.8
Quercetin	18.88	301	151	58	194	1.0	1.7
Isoxanthohumol	25.54	353	233	3	9	2.7	6.0
8-Prenylnaringenin	27.16	339	219	1	4	2.6	7.4
Xanthohumol	28.46	353	233	3	9	3.9	9.5
Cohumulone	29.64	347	278	4	13	2.3	1.8
Humulone	30.24	361	292	3	11	1.1	1.1
Colupulone	31.42	399	287	4	12	4.9	8.9
Lupulone	31.80	413	301	143	476	3.6	9.2

[*] µg/l Values
[*1] RT = Retention Time
[*2]LOD = Limit of Detection.
[*3]LOQ = Limit of Quantification.
[*4] Accuracy and precision were expressed as standard deviations %.

Table 2. Matrix effect (ME) in individual phenolic compounds in brewing by-products. The standard deviation were calculated on three replicates.

	Starting Malt		Wort		Spent Malt		Hop		Yeast		Beer	
Analyte	ME%	SD%	ME%	SD%	ME%	SD%	ME%	SD%	ME%	SD%	ME%	SD%
Gallic Acid	111	6.5	12	2.9	74	2.9	2	2.9	20	0.8	56	3.9
Catechin	122	0.6	92	1.6	84	3.6	86	0.2	81	6.7	84	4.4
4-CQA	141	7.2	132	1.6	103	3.7	114	2.4	105	5.9	97	0.4
3-CQA	136	4.2	135	3.1	100	0.1	109	3.7	118	5.9	120	0.8
4-Hydroxybenzoic acid	73	1.5	88	0.3	40	3.0	73	1.3	68	7.2	73	2.0
Epicatechin	119	8.1	128	0.1	98	6.3	101	1.4	112	5.9	87	0.1
Caffeic Acid	113	3.1	64	6.1	88	0.6	55	5.9	105	4.8	85	3.1
Vanillic Acid	120	0.1	61	8.4	92	9.1	125	5.0	52	0.6	118	0.3
Syringic Acid	147	3.9	117	3.4	106	3.0	115	3.5	95	7.9	133	2.1
trans-p-Coumaric Acid	106	1.2	64	1.1	59	1.8	53	1.3	56	2.8	91	1.1
trans-Ferulic Acid	131	0.3	183	6.9	94	2.7	60	9.3	82	1.9	79	6.2
Sinapic Acid	145	1.4	125	0.1	94	1.2	115	1.0	115	7.1	144	4.5
Quercetin	87	0.9	80	7.1	82	0.3	67	2.3	71	7.7	79	1.8
Isoxanthohumol	113	7.7	111	2.3	81	5.2	90	8.8	85	7.9	105	0.5
8- Prenylnaringenin	106	4.5	98	0.5	58	5.1	86	1.2	82	0.5	96	2.6
Xanthohumol	59	0.2	42	7.0	22	3.7	48	6.2	75	7.0	64	3.1

Cohumulone	127	0.6	52	2.0	171	2.6	87	1.8	112	1.0	69	6.7
Humulone	131	4.5	42	1.4	165	5.4	69	9.8	81	0.1	41	4.4
Colupulone	211	0.9	20	2.1	43	5.1	12	4.5	29	7.1	13	0.7
Lupulone	411	2.5	28	1.1	53	4.9	17	3.2	34	8.1	13	5.1

Table 3. Content in individual polyphenols in barley malt

	Mais		Special Black		Pilsen		Munich		Amber	
Analyte	µg/Kg	SD%	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%
Gallic Acid	1.4	2.4	3.2	0.5	3.9	4.0	5.6	0.2	5.8	5.4
Catechin	<LOQ	-	<LOQ	-	1.2	4.8	1.0	5.2	1.2	4.2
4-CQA	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
3-CQA	0.1	8.3	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
4-Hydroxybenzoic acid	1.1	6.5	2.8	1.2	1.9	0.8	3.4	1.8	1.4	2.2
Epicatechin	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
Caffeic Acid	0.2	1.7	0.6	2.7	0.7	2.9	1.1	4.6	0.9	3.6
Vanillic Acid	< LOQ	-	2.8	3.3	2.9	3.3	2.5	1.8	< LOQ	-
Syringic Acid	0.2	7.2	0.7	2.1	0.2	3.0	0.4	3.5	<LOQ	-
trans-p-Coumaric Acid	73.0	1.2	474.1	1.1	463.7	1.8	657.2	1.3	448.0	2.8
trans-Ferulic Acid	<LOQ	-	0.7	4.0	1.4	1.8	1.0	0.2	1.1	2.8
Sinapic Acid	0.2	2.3	<LOQ	-	0.1	2.3	<LOQ	-	0.1	3.1
Quercetin	0.1	5.8	0.3	-	0.3	2.3	0.3	3.1	0.3	4.9
Isoxanthohumol	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
8- Prenylnaringenin	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
Xanthohumol	0.1	4.2	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
Cohumulone	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-

Humulone	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
Colupulone	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
Lupulone	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
TOTAL (TPC*[1])	**76.4**		**485.2**		**476.3**		**672.6**		**458.7**	

[*] The mean value is expressed a µg/Kg with its standard deviation, respective to triplicate analyses.

[*1]TPC = total of individual polyphenol content.

Table 4. Content in individual polyphenols in brewing by-products (barley husks)

	Fiat lux		Triplo Malto		Alter		Maior		Ego		Ubi	
Analyte	µg/Kg	SD%	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%
Gallic Acid	8.3	2.2	8.9	4.9	7.5	0.2	11.4	5.6	9.7	3.8	7.4	2.4
Catechin	3.0	4.6	1.6	4.7	8.4	5.1	2.6	5.2	4.0	5.9	3.0	4.4
4-CQA	< LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
3-CQA	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
4-Hydroxybenzoic acid	4.5	7.5	13.4	1.3	7.7	2.8	17.0	1.8	4.0	2.2	5.8	2.1
Epicatechin	< LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ		<LOQ	-
Caffeic Acid	3.3	1.6	1.5	2.6	0.9	1.5	1.6	3.5	0.8	1.4	1.9	2.5
Vanillic Acid	< LOQ	-	< LOQ	-	<LOQ	-	< LOQ	-	< LOQ	-	2.2	0.3
Syringic Acid	< LOQ	-	0.2	3.4	0.3	3.0	0.2	3.5	<LOQ	-	0.4	2.1
trans-p-Coumaric Acid	904.4	1.2	471.9	1.0	413.19	1.8	581.3	1.3	321.2	2.8	947.5	1.1
trans-Ferulic Acid	<LOQ	-	4.0	6.9	12.4	2.7	1.1	4.3	<LOQ	-	<LOQ	-
Sinapic Acid	0.1	1.4	<LOQ	-	0.1	1.2	0.1	1.8	0.1	3.9	<LOQ	-
Quercetin	2.2	0.9	0.3	7.1	0.3	3.3	0.5	2.3	0.4	0.7	1.0	1.8
Isoxanthohumol	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
8- Prenylnaringenin	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
Xanthohumol	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
Cohumulone	0.1	0.6	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-

Humulone	0.3	4.5	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	3.4
Colupulone	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
Lupulone	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-
TOTAL (TPC*[1])	**926.2**		**501.8**		**450.8**		**615.8**		**340.0**		**969.2**	

[*] The mean value is expressed a µg/l with its standard deviation, respective to triplicate analyses.

[*1]TPC = total of individual polyphenol content.

Table 5. Content in individual polyphenols in intermediate product (worts)

	Fiat lux		Triplo Malto		Alter		Maior		Ego		Ubi	
Analyte	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%
Gallic Acid	< LOQ	-	< LOQ	-	0.3	6.0	< LOQ	-	0.3	0.6	< LOQ	-
Catechin	2.0	4.6	2.12	5.2	1.8	5.2	1.5	4.2	2.5	3.8	1.5	6.5
4-CQA	< LOQ	-	< LOQ	-	< LOQ	-	<LOQ	-	< LOQ	-	< LOQ	-
3-CQA	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-	<LOQ	-	< LOQ	-
4-Hydroxybenzoic acid	1.1	3.2	0.55	3.1	0.7	3.9	0.7	0.4	0.7	3.6	0.8	1.1
Epicatechin	< LOQ	-	0.01	5.3	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-
Caffeic Acid	1.3	7.8	0.68	2.4	0.3	2.1	1.00	5.7	0.7	3.2	0.3	4.3
Vanillic Acid	0.8	4.1	0.50	2.9	< LOQ	-	1.0	4.1	1.2	2.2	0.5	2.8
Syringic Acid	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-
trans-p-Coumaric Acid	448.0	3.0	1903.87	3.8	2036.0	3.7	280.6	3.3	125.3	0.3	1936.9	2.3
trans-Ferulic Acid	1.0	1.1	0.54	5.2	1.6	2.2	0.9	4.2	< LOQ	-	1.8	3.7
Sinapic Acid	0.5	2.0	0.19	3.3	0.4	5.7	0.2	7.9	0.2	5.6	0.2	5.3
Quercetin	0.1	3.1	0.10	2.6	0.1	2.8	0.1	2.7	0.1	3.1	0.1	4.6
Isoxanthohumol	< LOQ	-	< LOQ	-	< LOQ	-	0.1	4.1	< LOQ	-	< LOQ	-
8- Prenylnaringenin	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-

Xanthohumol	0.1	2.7	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-	0.03	4.3
Cohumulone	1.0	3.9	0.2	4.4	0.16	4.5	0.2	4.8	0.1	1.6	0.2	3.0
Humulone	0.3	1.4	0.5	3.3	0.46	2.1	0.5	2.6	0.4	3.5	0.5	2.4
Colupulone	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-
Lupulone	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-
TOTAL (TPC*[1])	**456.0**		**1909.3**		**2041.6**		**286.7**		**131.1**		**1942.8**	

* The mean value is expressed a µg/Kg with its standard deviation, respective to triplicate analyses.

*1TPC = total of individual polyphenol content.

Table 6. Content in individual polyphenols in brewing by-products (starting hops Perle and Saaz, spent hops)

	Hop Perle		**Hop Saaz**		**Fiat lux**		**Triplo Malto**		**Alter**		**Maior**		**Ego**		**Ubi**	
Analyte	µg/Kg	SD %	µg/Kg	SD %	µg/Kg	SD%	µg/Kg	SD%	µg/Kg	SD%	µg/Kg	SD%	µg/Kg	SD%	µg/Kg	SD%
Gallic Acid	83.0	4.	42.7	3.9	9.2	0.7	0.2	1.2	11.5	1.7	6.9	1.5	8.6	1.3	< LOQ	-
Catechin	65.6	3.1	78.0	5.6	1.5	6.2	1.4	3.9	2.1	5.7	1.3	6.1	2.3	4.2	1.3	2.3
4-CQA	56.3	6.4	49.3	0.1	0.3	0.9	0.2	2.2	0.2	2.1	0.2	2.7	0.1	2.2	0.2	1.0
3-CQA	10.3	4.1	7.3	0.4	0.1	6.0	0.4	3.0	0.5	0.5	0.1	1.9	0.1	6.4	0.2	3.0
4-Hydroxybenzoic acid	16.9	4.1	15.7	2.1	1.4	4.7	0.8	2.5	1.2	3.5	0.6	3.8	0.7	1.7	1.4	4.7
Epicatechin	1.1	3.2	0.6	4.8	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	0.1	0.3	<LOQ	-
Caffeic Acid	28.9	0.6	43.6	2.6	0.1	1.2	0.1	1.2	0.4	1.3	0.1	2.7	0.1	1.9	0.4	3.2
Vanillic Acid	0.9	5.6	1.1	3.4	1.0	0.4	0.3	2.1	0.5	1.7	0.4	0.1	0.7	3.1	0.4	3.6
Syringic Acid	0.6	3.5	0.5	0.7	0.2	4.3	<LOQ	-	0.3	5.1	<LOQ	-	0.2	3.3	0.2	2.3
trans-p-Coumaric Acid	17.2	0.1	22.5	2.4	<LOQ	-	<LOQ	-	0.1	2.3	<LOQ	-	0.1	2.8	0.1	2.2
trans-Ferulic Acid	30.6	0.9	28.2	5.4	3.7	2.9	2.2	3.6	4.2	0.2	3.6	0.9	1.3	2.2	4.4	4.1
Sinapic Acid	2.9	5.6	2.5	5.3	0.2	3.6	0.2	3.4	0.3	1.9	0.2	5.4	0.2	0.4	0.2	4.9
Quercetin	2.6	3.7	2.5	5.6	0.1	0.8	0.2	2.6	0.1	4.2	0.1	2.0	0.2	3.5	0.2	5.7
Isoxanthohumol	1.0	1.8	0.3	0.5	0.6	2.8	1.0	1.7	0.4	1.4	1.0	1.1	0.5	2.2	0.8	5.6
8- Prenylnaringenin	0.1	5.5	0.1	5.6	< LOQ	-	< LOQ	-	<LOQ	-	< LOQ	0.8	< LOQ	-	< LOQ	-

Xanthohumol	13.4	5.1	3.0	0.6	0.05	5.1	< LOQ	-	<LOQ	-	0.1	5.4	< LOQ	-	< LOQ	-
Cohumulone	16.5	3.9	7.9	0.2	0.99	1.2	<LOQ	-	0.7	1.0	<LOQ	-	1.5	6.4	<LOQ	-
Humulone	99.3	3.5	17.5	3.5	2.52	3.3	0.98	4.4	1.8	0.8	1.48	5.0	4.0	7.3	1.4	2.4
Colupulone	30.6	1.9	0.3	1.3	< LOQ	-	< LOQ	-	<LOQ	-	< LOQ	-	<LOQ	-	< LOQ	-
Lupulone	22.7	0.4	0.3	5.8	< LOQ	-	< LOQ	-	<LOQ	-	< LOQ	-	< LOQ	-	< LOQ	-
TOTAL (TPC*[1])	**500.3**		**323.8**		**22.0**		**8.0**		**24.4**		**16.0**		**20.6**		**11.2**	

[*] The mean value is expressed a µg/Kg with its standard deviation, respective to triplicate analyses.

[*1]TPC = total of individual polyphenol content.

Table 7. Content in individual polyphenols in brewing by-products (starting yeast and spent yeasts)

	Starting Yeasts		Fiat lux		Triplo Malto		Alter		Maior		Ego		Ubi	
Analyte	µg/Kg	SD%	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%	µg/kg	SD%
Gallic Acid	7.4	6.3	2.4	1.7	1.6	1.2	1.2	1.6	1.4	1.3	3.7	1.0	2.4	1.2
Catechin	< LOQ	-	3.5	4.0	6.7	4.4	6.0	3.2	1.6	3.6	4.7	3.2	2.3	3.1
4-CQA	< LOQ	-	0.6	0.2	0.7	0.2	0.4	1.0	0.3	0.2	0.2	0.1	0.4	0.2
3-CQA	< LOQ	-	0.2	2.3	0.2	2.9	0.1	2.7	0.1	2.2	0.1	1.0	0.1	2.0
4-Hydroxybenzoic acid	< LOQ	-	2.6	1.3	2.2	2.1	1.4	3.2	1.2	1.3	1.6	2.6	2.1	2.2
Epicatechin	< LOQ	-	<LOQ	-	0.2	0.2	0.1	0.8	0.2	1.2	0.9	1.6	<LOQ	-
Caffeic Acid	< LOQ	-	0.7	4.2	1.8	2.7	0.5	2.3	0.5	2.8	0.5	1.1	1.0	3.7
Vanillic Acid	0.3	1.4	3.0	2.7	3.4	1.1	2.9	0.7	1.3	1.9	2.6	3.9	2.9	1.2
Syringic Acid	0.6	2.3	0.6	3.9	0.4	3.7	0.6	3.6	0.3	2.2	0.6	2.2	0.4	4.3
trans-p-Coumaric Acid	< LOQ	-	0.3	1.2	6.2	2.3	0.1	0.4	0.1	2.9	0.2	2.5	0.5	4.0
trans-Ferulic Acid	0.1	2.6	2.5	1.7	11.3	2.3	5.2	3.3	2.4	1.2	1.3	2.9	3.1	1.0
Sinapic Acid	0.1	7.9	0.8	2.4	1.1	0.6	0.7	1.2	0.5	0.9	0.6	3.6	0.6	0.6
Quercetin	2.4	3.8	0.1	3.4	0.1	1.2	0.1	3.2	0.3	1.4	0.2	2.9	0.1	2.8
Isoxanthohumol	< LOQ	-	3.3	4.0	3.2	1.2	2.7	4.0	1.5	2.4	2.2	0.4	2.6	3.1
8- Prenylnaringenin	0.1	1.56	<LOQ	-	<LOQ	-	LOQ	-	<LOQ	-	<LOQ	-	0.1	2.1
Xanthohumol	< LOQ	-	0.1	1.2	<LOQ	-	0.07	1.8	0.2	1.8	0.1	1.1	0.1	1.2
Cohumulone	< LOQ	-	0.4	2.2	2.1	1.5	0.71	2.1	0.2	1.4	2.7	1.7	0.4	1.4

Humulone	< LOQ	-	0.6	4.0	2.6	1.9	1.1	2.3	0.3	2.9	3.6	0.2	0.5	3.7
Colupulone	< LOQ	-	< LOQ	-	12.9	2.6	< LOQ	-	<LOQ	-	<LOQ	-	< LOQ	-
Lupulone	< LOQ	**-**	< LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	< LOQ	-
TOTAL (TPC*[1])	**11.0**		**21.6**		**56.7**		**23.9**		**12.5**		**24.9**		**19.4**	

* The mean value is expressed a µg/Kg with its standard deviation, respective to triplicate analyses.

*1TPC = total of individual polyphenol content.

Table 8. Content in individual polyphenols in the six craft beers

	Fiat lux		Triplo Malto		Alter		Maior		Ego		Ubi Red	
Analyte	µg/Kg	SD%	µg/Kg	SD%	µg/Kg	SD%	µg/Kg	SD%	µg/Kg	SD%	µg/Kg	SD%
Gallic Acid	6.7	4.4	1.1	1.5	1.6	5.2	1.1	0.9	1.3	2.8	2.3	0.3
Catechin	4.1	3.1	27.1	5.7	34.4	3.5	12.5	6.6	24.5	1.4	2.7	0.4
4-CQA	2.3	3.6	3.2	5.0	3.1	4.4	2.8	4.2	2.8	1.2	2.9	5.75
3-CQA	0.7	2.5	1.3	4.3	1.6	4.0	1.2	3.6	0.5	3.2	0.8	4.5
4-Hydroxybenzoic acid	10.3	0.2	7.6	3.9	10.0	5.9	8.8	3.0	9.1	1.5	10.1	1.4
Epicatechin	0.9	3.2	5.9	5.2	5.5	0.2	2.0	5.4	4.7	6.1	0.6	2.3
Caffeic Acid	2.2	3.9	2.2	3.9	1.9	5.8	0.3	5.9	1.4	4.4	1.5	5.7
Vanillic Acid	13.2	1.7	9.2	5.7	14.2	2.3	7.9	5.1	10.9	8.7	13.4	0.2
Syringic Acid	1.0	2.2	1.7	1.7	1.4	1.0	0.9	3.1	1.7	3.1	0.8	4.7
trans-p-Coumaric Acid	1.0	5.3	3.2	6.0	0.82	1.7	4.7	7.0	0.6	2.6	2.2	4.6
trans-Ferulic Acid	7.2	2.8	15.8	5.9	10.4	4.6	<LOQ	-	8.1	4.1	13.9	5.0
Sinapic Acid	1.2	5.0	1.7	4.2	2.0	3.9	0.3	5.1	2.8	4.2	1.4	7.5
Quercetin	0.7	4.7	2.1	5.5	2.8	4.2	2.1	5.8	0.9	5.6	1.3	5.9
Isoxanthohumol	8.6	0.8	10.0	3.1	8.5	2.5	14.2	1.3	5.8	5.0	6.5	2.4
8- Prenylnaringenin	0.2	2.5	0.2	1.8	0.1	4.0	0.3	3.5	0.1	4.0	0.1	5.3

Xanthohumol	0.3	5.3	0.2	4.3	0.25	1.5	1.4	4.9	0.3	3.3	0.1	3.1
Cohumulone	1.0	1.4	1.2	3.3	1.5	2.0	1.3	6.1	0.9	3.1	1.2	7.1
Humulone	4.0	3.2	4.1	3.4	5.2	3.1	4.6	5.1	3.4	5.1	4.5	3.5
Colupulone	<LOQ	-	0.1	5.1	<LOQ	-	0.1	5.0	<LOQ	-	0.1	4.1
Lupulone	<LOQ	-	< LOQ	-	<LOQ	-	<LOQ	-	<LOQ	-	0.1	6.80
TOTAL (TPC*[1])	**65.6**		**97.8**		**105.3**		**66.0**		**79.8**		**66.4**	

[*] The mean value is expressed a µg/Kg with its standard deviation, respective to triplicate analyses.

[*1]TPC = total of individual polyphenol content.

Figure legend

Figure 1. Illustration of the craft beer brewing process.

Figure 2. Comparison of the ME% for the Ego Alter beer evaluated under different techniques. ME% was calculated from triplicate analyses and standard deviations are represented as error bars.

Figure 3. ME% in the six beers under study. ME% was calculated from triplicate analyses and standard deviations are represented as error bars.

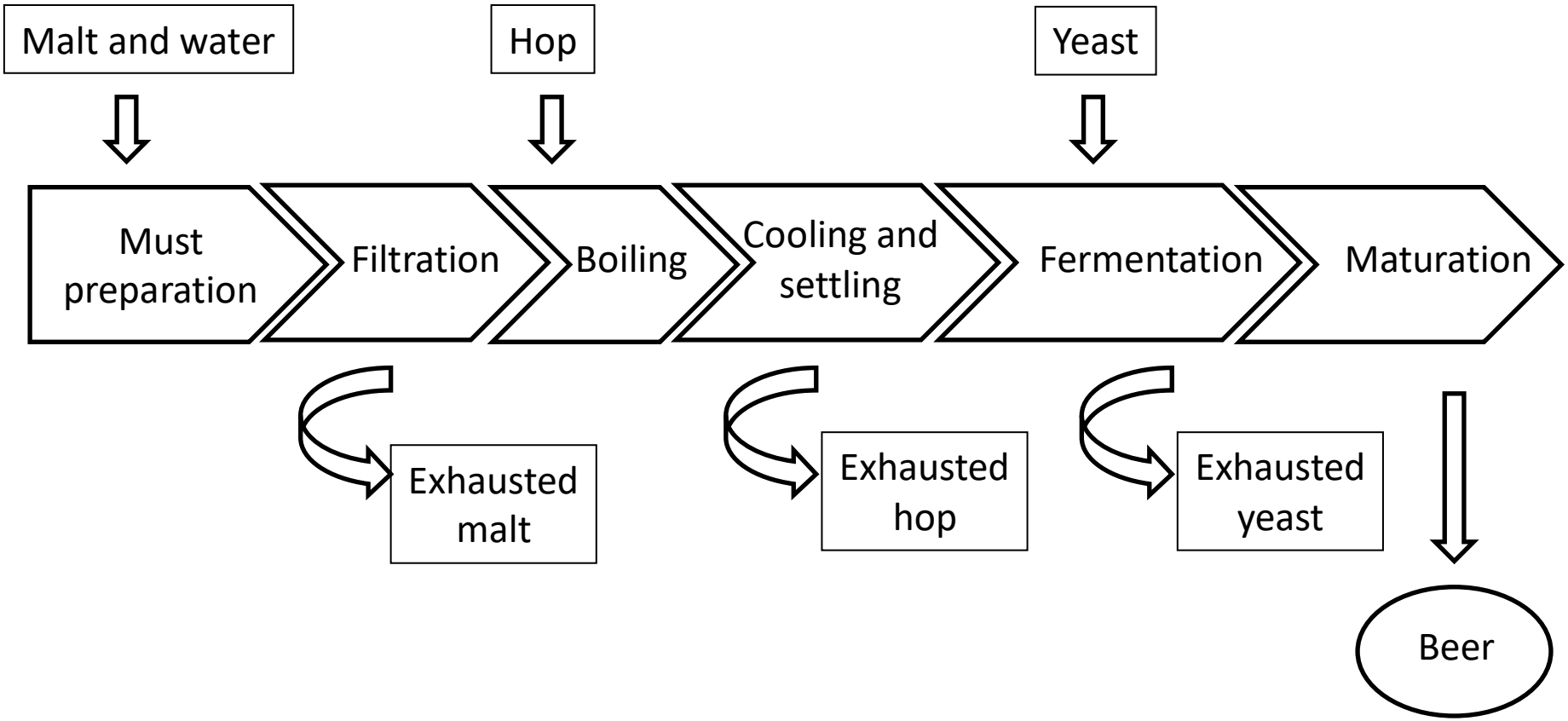

Figure 1. Illustration of the craft beer brewing process

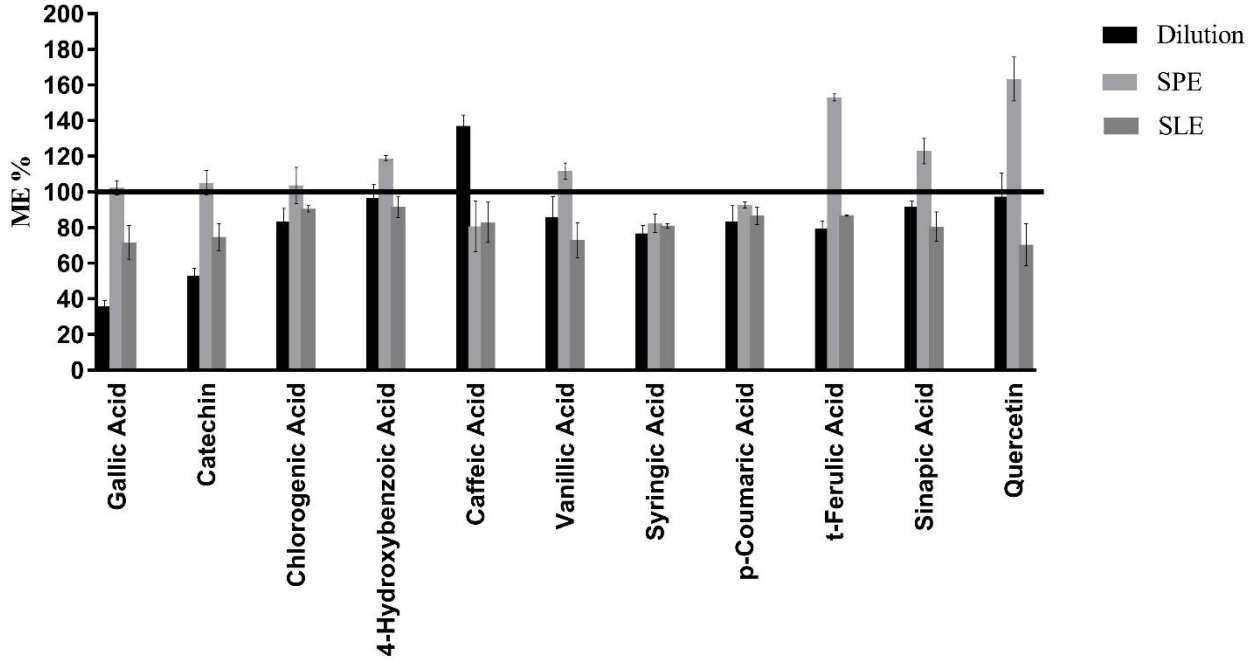

Figure 2. Comparison of the Matrix Effect percentage (ME%) for the Ego Alter beer evaluated under different techniques. ME% was calculated from triplicate analyses and standard deviations were represented as error bars.

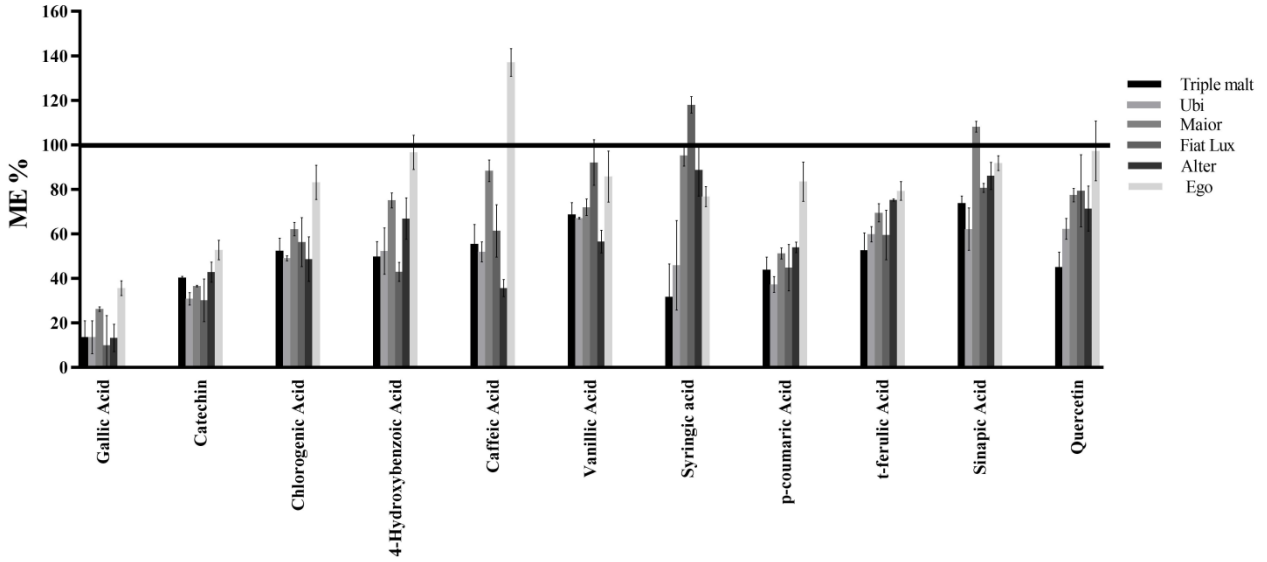

Figure 3. Matrix Effect percentage (%) in the six beers under study diluted 1:2. ME% was calculated from triplicate analyses and standard deviations were represented as error bars.